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THE RÔLE OF THE THYROID IN THE CALORIGENIC ACTION OF VITAMIN D[1]

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It has been shown that vitamin D in massive, but subtoxic, doses will enormously increase the metabolic rate of normal dogs and rats (1, 2). Landelius and Ljungkvist (6) have recently confirmed the earlier observations of Seel (7) that vitamin D will restore the rate of oxygen consumption to normal when it has been decreased in rickets. Goormaghtigh and Handovsky (3) have presented evidence of a thyrotropic influence of vitamin D and Gelfan (4) has shown that isolated muscles of frogs treated with vitamin D utilize more oxygen than do those from normal frogs. These latter experiments are unique in another respect, i.e., the demonstration of a response of a cold blooded form to vitamin D.

That the calorigenic effect is not due to an action on the parathyroids is indicated by the report of Reed, Steck and Miller (5) that parathyroid extract was wholly ineffective as a calorigenic agent in normal dogs.

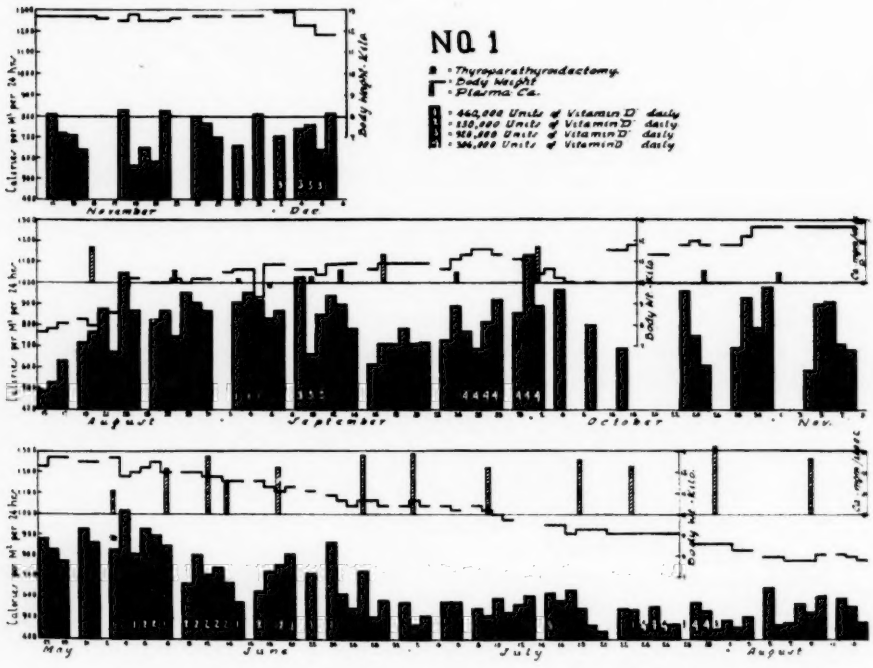
It is the purpose of this paper to report experiments on two dogs which demonstrate the importance of the thyroid in this reaction. The basal metabolic rate was determined on two normal dogs over a preliminary control period after which both were subjected to complete thyroparathyroidectomy. At autopsy no accessory thyroid tissue was found. It is, of course, possible that there may still have been microscopic rests of accessory tissue. But in any case, the total amount of thyroid tissue was *reduced* to a minimum. The results are described in the protocols and illustrated in the figure.

[1] The expenses of this investigation were in part defrayed by grants from the Wisconsin Alumni Research Foundation and from Mead Johnson and Company. The vitamin D in the form of viosterol (1,000,000 units per gram) was supplied by the latter.

A preliminary report of this work was read before the American Physiological Society, March 27, 1936.

Dog 1, young adult, male, 12.5 kilos (fig. 1).

The average metabolic rate for a period of 3 weeks before operation was 850 calories per square meter per 24 hours. Only 6 preoperative determinations are shown on the chart, but the variations before that were comparable to those charted. On June 3 a total thyroidectomy was done. No particular effort was made to remove the parathyroids, although it was ascertained later that the main bodies were all removed. No accessory thyroid tissue was found at autopsy. The concentration of total blood calcium was 11.11 mgm. per 100 cc. of heparinized plasma before the operation. There was a mild postoperative caloric reaction but in general the metabolic rate decreased progressively through June and July, approximately parallel with the decline in body weight to 7.75 kgm. on August 15. A diet of raw



beef was now substituted. With the prompt recovery of weight the metabolic rate increased to a normal level.

Oral administration of vitamin D was done in 5 periods, as follows:

	units
May 5 to June 14	4,830,000
July 15 to August 31	3,830,000
September 3 to September 11	4,140,000
September 25 to October 1	1,142,000
November 29 to December 6	3,220,000

During the period of weight decline to August 15 the metabolic rate fluctuated

between 500 and 600 calories/m^2./24 hours. The average from August 23 to September 3 was 893 cal. During the third period of vitamin D administration and the three succeeding days, the average was 875 cal.; from September 15 to 25, the average was 738; from September 25 to October 7, the average was 877.

After the operation the blood calcium remained high (14–20 mgm./100 cc.) until August 28. Thereafter it fluctuated between 7.8 and 15 mgm.

Dog 2, young adult male, 12.5 kilos.

The preoperative metabolic rate over 18 days averaged 930 cal./m^2./24 hours. June 16, complete thyroidectomy. There was a postoperative increase in the metabolic rate for 3 days, then a definite decline to 620 calories on August 15, at which time the weight was 9.5 kilos. On a raw meat diet the weight increased to 12 kilos by September 4. The total administration of vitamin for each of 4 periods was as follows:

	units
June 18 to 28	2,070,000 (no calorigenic effect)
July 15 to 31	4,444,000 (no calorigenic effect)
September 10 to 11	1,840,000 (no calorigenic effect)
September 25 to October 1	2,142,000 (see text)

During the period of weight gain from August 16 to September 4 the average metabolic rate was 880, but the fluctuations were very wide. From September 5 to 15 inclusive the average was 810 cal. During the last period of vitamin D administration and the subsequent 4 days the average rate was 842 cal. The average rate from October 28 to December 15 inclusive was 800 cal.

In the experiments on normal dogs the oral administration of amounts of vitamin D such as were given to both of these animals produced definitely, sustained augmentation of the metabolic rate within 3 to 5 days. The only suggestion of a calorigenic effect in either of these animals was seen in no. 1 on October 1 and in 2 during the week immediately following the fourth period of vitamin D administration when the rate was slightly higher than immediately before or after. In neither instance, however, were the effects anything like those seen in normal dogs with comparable doses of vitamin D.

The spontaneous variation occurring in these animals was very striking. It was much more difficult to get consistent results than in any normal dog we have ever trained. It would seem to be a valid assumption that this instability is a manifestation of the combination of the hypothyroid and hypoparathyroid states. The restoration of the metabolic rate concurrently with weight gain was a result that might have been predicted but was unexpected.

The marked variations occurring in no. 2 through August were correlated with a state of extreme nervous irritability that did not appear to be a manifestation of incipient tetany. While it is not impossible for tetany to occur with a high concentration of blood calcium such a result is unusual; especially when it is considered that at no time did either of these animals display any tetanic symptoms.

However, granting that some of the temporary fluctuations were due to the calorigenic effect of vitamin D, there certainly was no such response as occurred in dogs with normal thyroids. That no. 1 became intoxicated to a mild degree by the 4th period of vitamin D administration is indicated by the sharp decline in weight from September 27 to October 7. Past experience would lead one to expect that such intoxication would give a still greater augmentation of metabolism.

If one assumes that in the two instances cited there was a mild augmentation of metabolism, the mechanism by which it was produced must come in for consideration. Since no accessory thyroid tissue was found the total amount present must be so small that it seems unlikely that it could produce any effect.

The work of Gelfan suggests a possible effect on peripheral tissues. The effect was not unlike that observed in isolated muscle from thyroxinized warm blooded forms. But since there is no agreement that thyroxin produces such a calorigenic influence in cold blooded forms it cannot, at present, be assumed that the effects he obtained were due to thyroid stimulation. In that case the only other apparent explanation would be that the vitamin stimulates metabolism in peripheral tissues. Until some method is evolved for applying vitamin D directly to isolated peripheral tissues this point must remain unsettled.

That the calorigenic effect is not correlated with hypercalcemia was pointed out in the earlier work (1). This is still more definitely emphasized in these experiments when one considers that in both dogs the blood calcium was very high during the entire extent of the period of low metabolic rate.

Dog 1, after recovery of the original weight remained practically stationary while dog 2 had a second period of weight gain beginning September 16 and ultimately reaching 17.25 kgm. on November 15. However, there was no augmentation of metabolism correlated with this gain.

Whether the thyreotropic effect is due to direct action of vitamin D on the thyroid or to indirect action through the anterior pituitary is now under investigation.

While it has been maintained and is still maintained by some investigators that the administration of massive dosage of vitamin D has a direct effect on the parathyroids, it is clear from this and the preceding investigations that at least this particular effect of vitamin D does not involve the parathyroids. In fact, Steck, Reed and Miller (5) have shown that parathyroid extract tends to lower the metabolic rate, if anything. Furthermore, in one of their animals, long continued administration of parathyroid extract did not desensitize the animal to the subsequent calorigenic action of vitamin D.

Most investigators have assumed a rather limited physiological action of vitamin D. It seems probable, however, that its action on calcium-phosphorus metabolism is only one of many effects. We have considerable data supporting this view which will be presented later.

SUMMARY AND CONCLUSIONS

1. After complete thyroparathyroidectomy in two dogs there was a pronounced decrease in the metabolic rate correlated with loss in weight. With recovery of weight, the metabolic rate also was restored to nearly the original level.

2. Large doses of vitamin D produced no marked augmentation in the metabolic rate comparable to that produced in normal dogs.

3. This effect of vitamin D is not due to an action on the parathyroids.

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THE EFFECT OF EXPERIMENTAL HYPERTHYROIDISM ON CARBOHYDRATE METABOLISM[1]

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Although it is acknowledged by many that hyperthyroidism is associated with a profound disturbance in carbohydrate metabolism, the nature of the derangement is obscure. The associated syndrome of glycosuria, ketosis, low respiratory quotient and abnormal dextrose tolerance, as judged by the dextrose tolerance curve, resembles in many respects the syndrome observed in "hunger diabetes," pancreatic diabetes, and phlorhizin diabetes. This similarity is further emphasized by the fact that when hyperthyroidism develops in the course of diabetes mellitus, the diabetic condition is aggravated.

Such factors have led some investigators to assume that hyperthyroidism is associated with a disturbance either of the insulinogenic mechanism or the peripheral action of insulin. Others attribute the hyperthyroid syndrome to a relative diminution of carbohydrate oxidation subsequent to the depletion of glycogen stores. It is now well established that in animals the feeding of thyroid is followed by such a depletion (1). Cramer and Krause (2) interpreted this to mean that the storage of glycogen was defective in consequence of a decreased oxidation of carbohydrate. However, Sanger and Hun (3) after studying simultaneous curves of the respiratory exchange and blood sugar in a large series of patients concluded that there was no defect in the oxidation but rather an abnormality in the mobilization of glucose, namely, a defect in the mechanism by which glycogen is stored. This is in agreement with the observations of Cramer and M'Call (4) who found that thyroid-fed rats suffered not a decrease but an increase in the oxidation of carbohydrate. However, they attributed the changes to a primary defect in the glycogenic mechanism and a secondary increase in the oxidation of carbohydrate. From a series of respiratory studies on human subjects, Richardson, Levine and DuBois (5) and Johnston (6) concluded that there was no disturbance in the glycogen-storing mechanism but that the glycogenolytic mechanism was very unstable.

[1] Aided by the David May Memorial Fund.

In view of the fact that there are no direct observations on the utilization of carbohydrate in experimentally produced hyperthyroidism, we studied the rate of disappearance of glucose from the blood of eviscerated, hyperthyroid and normal rabbits. If either an increased rate of hepatic glycogenolysis "per se" or a decreased hepatic glycogenesis is the primary metabolic disturbance responsible for the diminution of glycogen observed in thyroid-fed animals, the rate of glucose removal by the muscles from the blood would be the same in both the normal and the hyperthyroid, eviscerated rabbit since the liver is not present to influence the blood sugar nor can tissue glycolysis be a significant factor (7). On the other hand, if an acceleration of carbohydrate utilization by the muscles is the primary disturbance, the rate of disappearance should be greater in the hyperthyroid than in the normal, eviscerated animal. By this means it should be possible to determine whether the utilization of carbohydrate or some derangement in the mechanism of glycogen storage is the primary disturbance in the hyperthyroid animal.

METHOD. Male New Zealand rabbits weighing from 1.5 to 2.0 kgm. in body weight were used in this study. Before the preliminary twenty-four hour fast, the animals were maintained on a commercial diet (Purina rabbit chow). Nembutal anesthesia was employed throughout all experiments in order to allow for the operative procedures and for the withdrawal of blood from the exposed femoral artery.

Evisceration was performed by a one-stage operation somewhat similar to that described by McMaster and Drury (8) for partial hepatectomy. The abdomen was opened and the intestines retracted to the right, exposing the aorta and inferior vena cava. The inferior mesenteric artery, the superior mesenteric artery and the coeliac axis were exposed and cut between ligatures. The gastro-intestinal tract was removed after cutting the rectum, esophagus and portal vein between ligatures. The arrangement of the rabbit liver into almost separate lobes permits the removal of practically the whole liver by simple ligation and excision. This is particularly simple in young rabbits where the lobes tend to be more distinct than in older animals. The fragments of liver that remain after this operation can be crushed between the fingers. The kidneys were removed after ligation of the hilus. The complete evisceration in this manner can be easily performed in about fifteen minutes.

In order to ascertain the completeness of the hepatectomy, we performed several eviscerations in rabbits employing the method of Markowitz, Yater and Burrows (9) where the inferior vena cava was replaced with a pyrex cannula. In several other instances, the two-stage operation of Drury (10) was performed. Comparison of our results with these procedures revealed no significant differences in the rate at which sugar was removed from the blood, and, hence, we subsequently confined ourselves to the above described procedure.

Hyperthyroidism was produced by the daily, oral administration of ten grain tablets of desiccated thyroid until a twenty to forty per cent loss of body weight occurred. This usually took from six to seven days.

Arterial blood samples were drawn immediately after the completion of the evisceration and at half-hourly intervals thereafter for one to two hours and the glucose content determined by the Somogyi modification of the Shaffer-Hartman method. In the majority of experiments with the hyperthyroid rabbits, glucose was administered at the end of the first hour, and, after a fifteen minute interval, the blood sugar decrement was studied for another hour. In all instances, the rate of glucose removal

TABLE 1

RABBIT NUMBER	DISAPPEARANCE OF BLOOD SUGAR FOLLOWING EVISCERATION		
	Immediate	30 minutes	60 minutes
	mgm. per cent	*mgm. per cent*	*mgm. per cent*
Control 1	50		25
Control 2	54		28
Control 3	59		37
Control 4	80	64	55
Control 5	85	65	59
Control 6	92	76	67
Control 7	101	83	74
Control 8	105		69
Hyperthyroid 1	48	15	
Hyperthyroid 2	78	38	10
Hyperthyroid 3	86	53	32
Hyperthyroid 4	87	56	18
Hyperthyroid 5	97	54	16
Hyperthyroid 6	104	62	25
Hyperthyroid 7	118	76	32
Hyperthyroid 8	87	51	29

during the second hour was, within the limits of experimental error, the same as that during the first hour.

RESULTS. Some of the results are detailed in table 1. Analysis of the control group reveals that the blood sugar dropped from 22 to 26 mgm. per cent in one hour whereas that of the hyperthyroid group fell from 54 to 86 mgm. per cent in the same interval of time. The average decrease in the blood sugar per hour was 26.5 mgm. per cent, with a standard deviation of ± 3.85 mgm. for the control group, and 70.7 mgm. per cent, with a standard deviation of ± 11.09 mgm. for the hyperthyroid group. Because of the difference in blood sugar level and the fact that the fall of the blood sugar was determined by the initial level, we computed the

rate of disappearance as the *per cent drop from the initial level.* The average sugar disappearance in one hour was 35.6 per cent for the control group and 75.5 per cent for the hyperthyroid group (fig. 1). In some instances, the rate of glucose disappearance from the blood of the hyperthyroid rabbits was so rapid that we obtained very low initial blood sugar levels and it was expedient to administer glucose within one hour to prevent the death of the animal.

Discussion. It is obvious from the above data that the extrahepatic tissues of the hyperthyroid rabbit remove glucose from the blood at a much greater rate than do those of the normal animal. Since the observations of Andrus and McEachern (7) indicate that the tissues of the hyperthyroid animal do not have a significantly increased glycolysis, it becomes probable that the increased rate of blood glucose removal which such animals exhibit is due to an increase in carbohydrate utilization. Furthermore, since the muscles of thyroid-fed animals are characterized by a marked depletion of glycogen, the glucose is probably used for purposes other than storage. Thus we are led to conclude that there is a marked acceleration in carbohydrate oxidation in the tissues of the hyperthyroid animal, and that the increased oxygen utilization of the intact hyperthyroid animal or of isolated tissues from such an animal is due to this increased rate of carbohydrate oxidation.

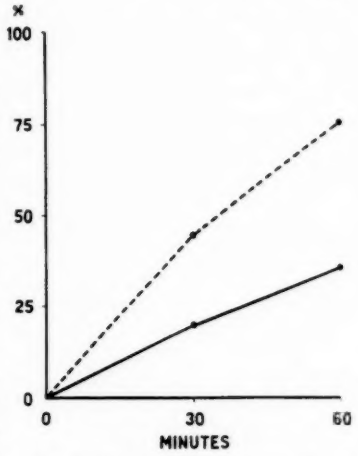

Fig. 1. Graphic comparison of the rates of disappearance of the blood sugar after complete evisceration in normal (solid line) and hyperthyroid (broken line) rabbits. Each curve represents the average per cent drop from the initial blood sugar level.

These observations exclude the possibility that the primary disturbance of the carbohydrate metabolism in hyperthyroidism is some defect in the glycogen storing mechanism of the liver, or a diminution of carbohydrate utilization by the extrahepatic tissues. The decreased liver glycogen content that is commonly observed in thyroid-fed animals is probably a secondary phenomenon consequent to 1, an increased utilization of carbohydrate by the liver itself with the onset of hyperthyroidism, and 2, an increased rate of removal of glucose from the liver by the extrahepatic tissues (glycogenolysis). A similar increase in hepatic glycogenolysis is known to occur in pancreatic and phlorhizin diabetes. In the former, Major and Mann (11) have demonstrated that glycogen can be synthe-

sized in the liver but that it cannot be retained unless insulin is administered, indicating that in this condition there is an inability of the cells to hold glycogen, so that the rate of glycogenolysis exceeds the rate of glycogenesis. In phlorhizin diabetes, where the renal threshold for glucose is lowered, an accelerated hepatic glycogenolysis occurs in response to the hypoglycemia which results from the loss of glucose via the kidneys. Thus

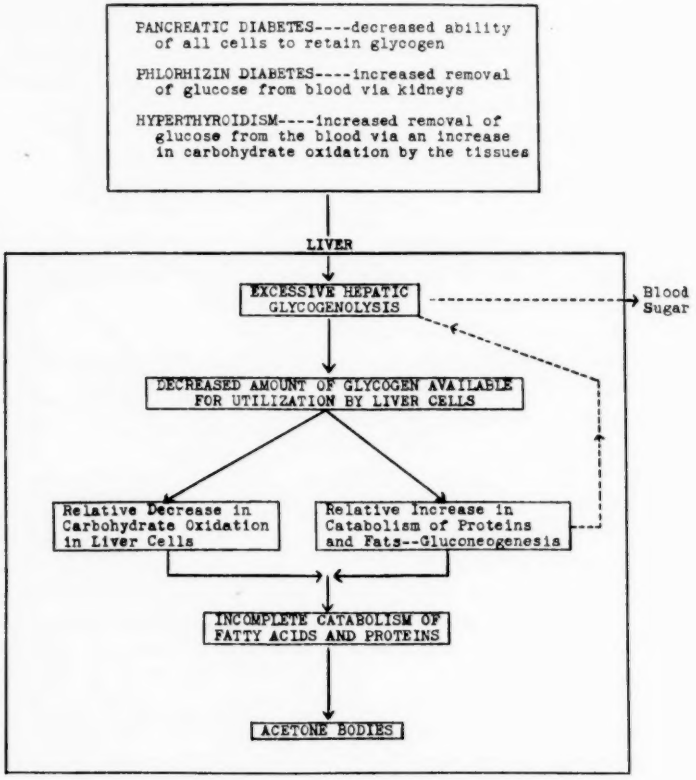

Fig. 2

in these three conditions an accelerated hepatic glycogenolysis is a common factor, being primary in pancreatic diabetes and secondary in phlorhizin diabetes and in hyperthyroidism.

In hyperthyroidism, as in the other conditions discussed above, ketosis is a frequent occurrence especially after a relatively short period of starvation. This cannot be attributed to a decrease in the utilization of carbohydrates by the extrahepatic tissues since our data indicate that the re-

verse actually occurs in hyperthyroidism. Furthermore, it is obvious from previously reported studies that the muscles do not contribute to the accumulation of ketones in the blood and that the liver is probably the only site of ketone body formation (12). The fact that starvation "per se" ("hunger diabetes") is not associated with a decrease in the utilization of glucose by the extrahepatic tissues lends support to this conclusion (13). These observations are in accord with a tentative hypothesis which we have previously presented in explanation of the mechanism responsible for ketone formation (12, 14). According to this hypothesis (fig. 2), a decrease in liver glycogen is tantamount to a decrease in the amount of carbohydrate available for oxidation by the liver itself (a relative decrease in carbohydrate oxidation in the liver). This is compensated for by an acceleration in the catabolism of fat and protein (gluconeogenesis). The oxidation of fatty acids coincident with a relative insufficiency of carbohydrate oxidation is incomplete and ketone bodies are formed. From this point of view, the same mechanism is responsible for ketone formation in all conditions associated with a depletion of liver glycogen (fig. 2).

The fact that the development of hyperthyroidism during the course of diabetes mellitus results in an aggravation of the diabetic condition is to be expected from the above considerations. Because of the pancreatic deficiency, the liver cannot retain glycogen and both a relative decrease in carbohydrate utilization by the liver and gluconeogenesis occur. The glucose arising from the latter process is discharged into the blood resulting in a hyperglycemia. With the onset of hyperthyroidism, an increase in the utilization of carbohydrate occurs in all tissues, leading to a more complete exhaustion of liver glycogen. This must result in a further increase in the rate of fat and protein catabolism by the liver, and, consequently, an exaggeration of the condition observed in each disease alone.

SUMMARY AND CONCLUSIONS

1. The utilization of carbohydrate by the extrahepatic tissues of the thyroid-fed rabbit is much greater than that of normal animals.

2. Since the liver is not present to influence the blood sugar, the increased utilization of glucose by the extrahepatic tissues of the eviscerated, hyperthyroid animal is not secondary to any defect in either the glycogenic or glycogenolytic mechanism of the liver.

3. It is suggested that the similarity between the hyperthyroid and diabetic syndromes is due to the accelerated glycogenolysis which is common to both conditions. This acceleration is probably a primary phenomenon in diabetes and a secondary one in the hyperthyroidism.

We are indebted to Miss Dorothea Hamm for technical assistance.

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A STUDY OF THE BLOOD SUGAR OF THE ADRENALECTOMIZED DOG

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Britton and Silvette (1932–1935) in a series of studies on rats, cats, marmots, opossums and dogs have vigorously championed the view that the adrenal cortex is a fundamental factor in regulating the metabolism of carbohydrate. According to them, ablation of the cortex leads to rapid depletion of the glycogen stores, the animal loses the ability to mobilize glycogen, the blood glucose falls to extremely low levels and the animal dies in hypoglycemic convulsions. They state, moreover, that injection of adrenal cortical hormone markedly elevates the blood glucose of the intact animal. (The extensive literature on this subject has been adequately reviewed by Wyman and Walker, 1929, and Britton, 1930 and subsequent publications.)

However, not all investigators who have studied the rat and cat agree that hypoglycemia is a constant finding in the adrenalectomized animal or that it plays an important rôle in adrenal insufficiency (Hartman, 1933; Zwemer and Sullivan, 1934).

In recent years those workers who have employed the dog agree that alterations of blood glucose levels in this species following ablation of the adrenals, are of no great significance in so far as the symptom complex resulting from adrenal removal is concerned (Rogoff and Stewart, 1926; Harrop et al., 1933, 1935; Swingle, Pfiffner, Vars and Parkins, 1934; Kendall, 1935).

The present study is concerned solely with the blood sugar level of the adrenalectomized dog subjected to various experimental procedures. The blood glucose determinations were made on tungstic acid filtrates with the Somogyi modification of the Shaffer-Hartman reagent and the blood samples taken from the femoral artery. In order to conserve space, only the terminal values are given in the tables, although the blood sugar was followed at intervals throughout the insufficiency and recovery periods. The arterial pressures were obtained by use of the needle puncture method (Parkins, 1934).

Blood glucose of adrenalectomized dogs permitted to develop adrenal insuf-

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ficiency by withholding cortical hormone. A total of twenty-eight cases of uncomplicated insufficiency exhibiting mild to severe symptoms was studied. Since the degree of insufficiency can easily be judged by the level of arterial pressure, these determinations are included in the table.

The data are presented in abbreviated form in table 1. Study of this table reveals that the bilaterally adrenalectomized dog does not usually exhibit marked deviation of the blood glucose from normal. Only a relatively few cases have come under our observation in which really significant lowering of the blood glucose occurs. (Cases 8, 13, 26 and 28,

TABLE 1

The blood glucose of bilaterally adrenalectomized dogs

EXPERIMENT NUMBER	GLUCOSE ON DAY EXTRACT WAS WITHHELD		SEVERE INSUFFICIENCY. CORTICAL HORMONE INJECTED IMMEDIATELY AFTER BLOOD SAMPLE TAKEN		EXPERIMENT NUMBER	GLUCOSE ON DAY EXTRACT WAS WITHHELD		SEVERE INSUFFICIENCY. CORTICAL HORMONE INJECTED IMMEDIATELY AFTER BLOOD SAMPLE TAKEN	
	Bp.	Glucose	Bp.	Glucose		Bp.	Glucose	Bp.	Glucose
	mm. Hg	*mgm./ 100 cc.*	*mm.* Hg	*mgm./ 100 cc.*		*mm.* Hg	*mgm./ 100 cc.*	*mm.* Hg	*mgm./ 100 cc.*
1	100	87	46	83	15	82†	73	68*	66
2	105	76	50	81	16	107	90	56	87
3	98	84	53	85	17	105	80	48	102
4	104	94	50	83	18	98	84	45	69
5	106	82	48	78	19	92	89	50	89
6	103	84	56	80	20	102	80	46	73
7	96	80	36	83	21	108	100	48	89
8	104	77	42	58	22	98	75	60	75
9	100	87	43	89	23	102	78	72	97
10	106	87	70*	87	24	100	80	44	75
11	96	73	60	80	25	98	80	44	80
12	98	75	54	70	26	104	87	53	59
13	83†	71	58	59	27	100	69	55	62
14	104	78	50	73	28	98	69	50	59

* Mild insufficiency.

† Subminimum maintenance dose of extract.

table 1, exhibited the most drastic reductions we have noted.) These are offset by other dogs in insufficiency which showed rises in blood sugar when the animals were in collapse, e.g., cases 2, 11, 17 and 23. Hypoglycemia is an uncommon occurrence in the adrenalectomized dog allowed to develop uncomplicated insufficiency by simply depriving him of cortical hormone. Lowering of the blood sugar levels can, however, be induced in these animals by various experimental procedures which as a general rule do not decrease the blood glucose of the dog with intact adrenals. This fact is illustrated in the following section.

Blood glucose changes following 1, *a single stage bilateral adrenalectomy, and* 2, *muscle trauma to the adrenalectomized dog.* Removal of both adrenals at a single stage is a severe operation in the dog and leads to rapid onset of shock and collapse. It has been the experience of workers in this laboratory (Swingle and Parkins, 1934) that dogs so treated rarely survive longer than 24 to 36 hours. Most of the dogs die within the first 24 hours following gland ablation unless treated with cortical hormone or large doses of concentrated sodium chloride and hormone. The serum electrolyte changes and altered fluid distribution occurring in these animals are similar, however, to those observed in uncomplicated adrenal insufficiency following extract withdrawal. The method of operation has been previously described and need not concern us here. Table 2 gives the pertinent data

TABLE 2

Changes in blood glucose following bilateral adrenalectomy at a single stage operation

EXPERIMENT NUMBER	BEFORE OPERATION		SHOCK AND COLLAPSE 10–26 HOURS AFTER OPERATION		REMARKS
	Bp.	Glucose	Bp.	Glucose	
	mm. Hg	*mgm./ 100 cc.*	*mm.* Hg	*mgm./ 100 cc.*	
1	117*	82	42	64	Recovery on hormone treatment
2	108	82	40	84	No treatment given. Death
3	106	84	54	64	No treatment given. Death
4	104	84	43	44	Injected with saline. Death
5	120*	87	56	80	Injected with saline plus extract. Recovery
6	124*	86	45	75	Injected with saline. Death
7	120*	87	45	66	Injected with saline plus extract. Recovery
8	118*	84	44	43	Injected with saline. Death

* Animals untrained for blood pressure work. Remainder of dogs trained.

on the blood glucose changes following removal of both adrenals at a single stage operation where no treatment of any kind was given or else the treatment was begun after the glucose sample had been taken.

In general the blood glucose tends to fall following the single stage bilateral operation, but with the exception of cases 4 and 8 (table 2) significant hypoglycemic levels were not reached even at death. Several of the dogs revealed negligible changes in blood sugar (expts. 2 and 5) when in profound shock and collapse. It would seem that the bilateral adrenalectomy at a single stage, by suddenly depriving the animal of all adrenal tissue, plus the trauma and shock of the double operation, does in some cases upset the balance of endocrine and possibly other non-hormonal factors regulating carbohydrate metabolism and hypoglycemic changes

may result. However, such changes are by no means constant, as shown in table 1. The adrenalectomized dog not receiving hormone treatment rarely presents significant changes in blood sugar even when moribund from insufficiency, when trauma and shock are not complicating factors.

It is of interest in this connection, to note that the tendency toward hypoglycemic changes which the animals sometimes exhibit can be con-

TABLE 3

Effect of adrenalin injections upon the blood glucose of dogs bilaterally adrenalectomized at a single stage operation

EXPERIMENT NUMBER	BEFORE OPERATION		TIME AFTER OPERATION	AFTER OPERATION		SYMPTOM	REMARKS
	Bp.*	Glucose		Bp.	Glucose		
	mm. Hg	*mgm./100 cc.*	*hours*	*mm. Hg*	*mgm./100 cc.*		
1	116	66	26	55	93	Shock, collapse	Adrenalin, 4.8 cc. 1:10,000 every 4 hours, subcutaneously. Death
2	118	84	22	40	69	Shock, collapse	Adrenalin, one injection 5.8 cc. 1:10,000. Extract injected. Recovery
3	115	78	16	60	80	Shock symptoms	Adrenalin, one injection 5.8 cc. 1:10,000. Extract injected. Recovery
4	112	84	27	28	73	Collapse	Adrenalin. Large doses every few hours. Death
5	120	84	15	34	48	Collapse	Adrenalin, 24 cc. 1:10,000 given in divided doses over 15-hour period. Death
6	121	84	26	58	71	Marked symptoms	Adrenalin 21 cc. 1:10,000 given in divided doses over 26-hour period. Death
7	110	80	25	36	98	Collapse	Adrenalin. Large doses every few hours. Death
8	116	87	19	45	75	Collapse	Adrenalin, 10 cc. 1:10,000 given in 2 doses over 19-hour interval. Death

* Animals not thoroughly trained for blood pressure determinations.

trolled by injecting adequate amounts of adrenalin. Such treatment, in the absence of cortical hormone, does not prolong the life-span of the animal, nor does it alleviate the shock symptoms, but if given in sufficient doses adrenalin does elevate the blood sugar to levels higher than the normal for the animal or else maintains it within the range of normal. Dogs so treated die in shock with a normal or elevated blood glucose. The essential data regarding this type of experiment are given in table 3. Only

those cases showing a definite tendency toward hypoglycemia were used for adrenalin injections. One animal failed to respond to the injections.

The amount of adrenalin required to bring about changes in blood glucose in this type of experimental animal is greater than that requisite for induction of similar changes in the normal intact animal. This is not peculiar to the adrenalectomized dog for it has been demonstrated by Cope

TABLE 4

Effect of muscle trauma upon the blood glucose of the healthy, vigorous, adrenalectomized dog

EXPERIMENT NUMBER	TIME	GLUCOSE	SYMPTOMS	Bp.	REMARKS
		mgm./100 cc.		*mm. Hg*	
1	10:20 a.m.	73	Normal	94	Traumatized
	1:10 p.m.	69	Deep shock	42	Death. No treatment given
2	8:45 a.m.	84	Normal	100	Traumatized
	12:15 p.m.	127	Shock		
	2:10 p.m.	213	Deep shock	38	Death. No treatment given
3	9:30 a.m.	71	Normal	98	Traumatized
	4:02 p.m.	73	Deep shock	32	Death within hour after sample taken. No treatment
4	9:50 a.m.	69	Normal	110	Traumatized
	2:45 p.m.	69	Weak	60	
	11:40 p.m.	59	Shock	52	Injection cortical hormone. Recovery
5	10:50 a.m.	82	Normal	110	Normal health and vigor. Traumatized
	7:30 p.m.	64	Normal	84	
	10:00 p.m.	50	Shock	50	Injection cortical hormone. Recovery
6	10:00 a.m.	80	Normal	108	Traumatized
	11:10 a.m.	80	Normal	68	
	3:00 p.m.	50	Shock	47	Injection cortical hormone. Recovery

and Marks (1934), that the effect of adrenalin injections in inducing hyperglycemia is greatly diminished following hypophysectomy. It is evident, however, that the shock resulting from the single stage bilateral adrenalectomy in the dog is not due to hypoglycemia.

In an earlier paper (Swingle and Parkins, 1935) it was observed that the shock syndrome following muscle trauma to healthy, vigorous, adrenalec-

tomized dogs on maintenance doses of cortical hormone was occasionally complicated by considerable reduction in the level of blood sugar. The data from this type of experiment are recorded in table 4. The blood sugar changes were quite variable. Some animals even at death reveal no significant changes, others show elevation and still others sharp decreases.

TABLE 5

The blood glucose of the shocked adrenalectomized dog treated with adrenalin

EXPERIMENT NUMBER	TIME	GLUCOSE	SYMPTOMS	Bp.	REMARKS
		mgm./100 cc.		*mm. Hg*	
1	10:20 a.m.	80	None	110	Normal health and vigor. Muscle trauma
	1:00 p.m.	80	None	98	
	3:30 p.m.	111	Marked	56	Injected with 6.4 cc. 1:10,000 adrenalin at 3:00 p.m.
	3:55 p.m.	202	Collapse	36	Died few minutes later
2	9:30 a.m.	84	None	103	Normal health. Traumatized
	2:00 p.m.	91	Slight	84	Injected with 5.8 cc. 1:10,000 adrenalin at 11:00 a.m., 5:00 p.m. and 7:30 p.m.
	7:10 p.m.	115	Mild shock	65	
	9:30 p.m.	89	Deep shock	46	Injection cortical hormone. Recovery
3	9:00 a.m.	89	None	109	Normal health. Traumatized
	4:30 p.m.	105	Mild shock	62	Injected with 5 cc. 1:10,000 adrenalin at 10:30 a.m. and 3:00 p.m.
	8:25 p.m.	96	Deep shock	42	Injection cortical hormone. Recovery
4	9:30 a.m.	82	None	103	Normal health. Traumatized
	1:30 p.m.	75	Moderate	70	
	5:00 p.m.	64	Marked	60	Injected with 5.5 cc. 1:10,000 adrenalin at 5:15 p.m. and again at 8:40 p.m.
	10:10 p.m.	91	Deep shock	48	Injection cortical hormone. Recovery

It proved to be a simple matter to control the level of blood sugar in these traumatized dogs by injecting adequate amounts of adrenalin. The blood glucose was thereby elevated above the normal but this was without effect upon the shock symptoms and it was necessary to inject cortical hormone in order to save the animals. Table 5 gives the essential data.

These experiments afford a probable explanation for the occasional abrupt decline in blood sugar following bilateral adrenalectomy at a single stage. The trauma incident to the double operation is apparently a contributing factor to the fall in blood sugar in these animals.

Effect of intravenous and intraperitoneal injections of large amounts of cortical hormone upon the blood sugar of the adrenalectomized dog. Six animals were studied with the view of determining whether or not injections of large amounts of cortical hormone have any significant effect upon the blood sugar level of the adrenalectomized dog prostrate from severe insufficiency and the healthy, vigorous dog lacking adrenals. Table 6 gives the pertinent data. The changes in blood sugar levels in both types of experimental animal do not appear to be important. Some of the animals may exhibit a few milligrams rise in blood glucose shortly after injection, whereas others do not show such changes. It will be observed that the amounts of hormone administered were considerable. Britton et al. (1932) and Zwemer and Sullivan (1934) maintain that the blood sugar is markedly elevated even in the normal animal when large doses of cortical hormone are injected. Harrop et al. (1933) were unable to confirm these observations. Our data agree with those of Harrop. In drawing conclusions from this type of experiment it should always be borne in mind that cortical extracts contain traces of adrenalin. The adrenalin content of the stock extracts employed in this laboratory varies between 1:500,000 and 1:1,000,000 as determined by blood pressure assay.

Blood glucose changes in the adrenalectomized dog in shock and collapse following intraperitoneal injections of isotonic glucose. The writers have elsewhere presented cogent reasons for assuming that the dog in insufficiency and the animal in collapse following intraperitoneal injections of glucose are physiologically comparable (Swingle, Parkins and Taylor, 1936, in press). If this assumption is valid then the blood sugar of the glucose-injected animals during prostration becomes of great significance. The data relating to this set of experiments are presented in table 7. It is obvious that the intraperitoneal injections of glucose raises the blood sugar but at the same time throws the animal into shock and collapse from dehydration. The recovery of these animals depends, not on the level of blood glucose, but whether or not normal internal osmotic relations are reëstablished either by injections of cortical hormone or concentrated sodium chloride.

Blood sugar of the adrenalectomized dog during oestrus (pseudopregnancy) and not receiving cortical hormone. The essential data obtained from study of four representative cases are shown in table 8. The animals employed were brought into oestrus by daily injection of extract of menopause urine[2]

[2] We are indebted to Dr. J. A. Morrell of E. R. Squibb and Sons for generous supplies of extract of menopause urine.

(25–50 units daily) for 6 to 10 days. Following the onset of oestrus the bitches were bred to a vasectomized male and the daily injections of cortical hormone and menopause urine extract discontinued. Blood sugar samples were drawn at 7 to 10 day intervals throughout the experiments.

TABLE 6

Effect of intravenous injection of large doses of cortical hormone upon the blood glucose of the adrenalectomized dog

EXPERIMENT NUMBER	TIME	GLUCOSE	SYMPTOMS	Bp.	REMARKS
			Adrenal insufficiency		
		mgm./100 cc.		*mm. Hg*	
1	11:45 a.m.	80	Severe insufficiency	44	34 cc. extract (3 cc./kgm.)
	1:50 p.m.	82			
	3:20 p.m.	84			
2	4:10 p.m.	82	Severe	48	28.8 cc. extract (3 cc./kgm.)
	8:00 p.m.	78			
3	12:00 m.	64	Mild insufficiency	66	30 cc. extract (3 cc./kgm.)
	2:00 p.m.	71			
	4:00 p.m.	80	None	88	
4	10:00 a.m.	89	Severe	50	26 cc. extract (3 cc./kgm.) 4/20/35
	12:00 m.	89			
	1:00 p.m.	96			
	2:00 p.m.	93	Slight improvement	57	
	10:00 a.m.	96	Marked improvement	76	4/21/35
			Normal health		
5	10:20 a.m.	64	None	97	12 cc. extract (1 cc./kgm.)
	12:15 p.m.	73	None	100	
6	10:00 a.m.	84	None	109	30 cc. extract (3 cc./kgm.)
	12:30 p.m.	89			
	2:30 p.m.	87			
	4:30 p.m.	84	None	107	

During the period of normal health and vigor which in our experience may vary from 40 to 60 days, the blood sugar levels tend to be higher than normal. There is some fluctuation near the end of the pseudopregnant period, however, and in one case (table 8) the blood glucose at the termina-

tion of the experiment when the animal was showing symptoms of insufficiency was somewhat below normal.

The explanation of this elevation of the blood sugar levels during oestrus and pseudopregnancy in the dog is not clear but may possibly have some

TABLE 7

Blood glucose changes in the healthy, vigorous, adrenalectomized dog thrown into shock and collapse by intraperitoneal injections of isotonic glucose

EXPERIMENT NUMBER	BLOOD SUGAR BEFORE GLUCOSE INJECTION		SHOCK AND COLLAPSE 1-9 HOURS AFTER GLUCOSE. HORMONE INJECTION IMMEDIATELY AFTER SAMPLE		REMARKS
	Bp.†	Glucose	Bp.	Glucose	
	mm. Hg	*mgm./ 100 cc.*	*mm.* Hg	*mgm./ 100 cc.*	
1	94	73	50	100	Extract injection. Recovery
2	98	64	40	170	No extract. Death
3	102	80	44	131	Extract injection. Recovery
4	103	87	50	102	Extract injection. Recovery
5	84*	72	26	96	No extract. Death
6	90	80	50	82	Extract injection. Recovery
7	100	89	62	140	Extract injection. Recovery

* Subminimum maintenance dose of extract.

† The dogs were thoroughly trained for blood pressure work.

TABLE 8

Blood glucose of adrenalectomized dogs during period of oestrus (and pseudopregnancy) and not receiving cortical hormone

DOG NUMBER	DATE	GLUCOSE BEFORE EXTRACT DISCONTINUANCE	DAYS OFF EXTRACT	GLUCOSE, ANIMAL IN INSUFFICIENCY*	Bp.	REMARKS
		mgm./ 100 cc.		*mgm./ 100 cc.*	*mm.* Hg	
1	2/23/36	82	43	96	56	Severe insufficiency. Extract injected
2	2/11/36	84	58	89	75	Mild insufficiency. Extract injected
3	4/17/35	76	40	60	50	Severe insufficiency. Extract injected
4	3/ 4/36	100	52	87	48	Severe insufficiency. Extract injected

* During the period of normal health the blood sugar was higher than these terminal figures indicate.

relation to the anterior pituitary. The work of Houssay and Biassotti (1931), Barnes and Regan (1933), Long and Lukens (1936) and others definitely shows that the anterior pituitary is intimately concerned with

carbohydrate metabolism. The drastic fall in blood sugar levels following hypophysectomy in the monkey has been recently demonstrated by Smith (1936).

SUMMARY AND CONCLUSIONS

1. The healthy, vigorous, adrenalectomized dog permitted to develop severe insufficiency by withholding cortical hormone does not usually exhibit significant deviations of the blood glucose from normal.

2. The dog bilaterally adrenalectomized at a single stage operation and the traumatized adrenalectomized animal may show sharp reductions in the blood sugar when in collapse. The blood glucose changes in these types of experimental animal are, however, inconstant and extremely variable.

3. Adrenalin, injected into such animals as reveal a tendency for the blood glucose to fall when subjected to the procedures mentioned above, raises the blood sugar to normal or considerably above normal. The high glucose has no effect upon the shock symptoms.

4. Intravenous or intraperitoneal injections of large amounts of cortical hormone of high unitage have no significant effect upon the blood sugar level of either the healthy, vigorous, adrenalectomized dog, or the animal prostrate from insufficiency.

5. Intraperitoneal injections of isotonic glucose into healthy, vigorous, adrenalectomized dogs on a maintenance dose of hormone induces shock and collapse. Animals so treated die (unless injected with cortical hormone or concentrated salt) with blood sugar levels far above the normal.

6. The adrenalectiomized bitch in oestrus (pseudopregnancy) maintains herself in normal health for 40 to 60 days without cortical hormone. During this interval the blood glucose is generally higher than normal.

7. The adrenal cortical hormone *per se* is apparently not directly concerned with the metabolism of carbohydrate, at any rate, in so far as this is reflected by changes in the blood glucose levels of the adrenalectomized dog.

8. Hypoglycemia is not a significant factor in adrenal insufficiency in this species.

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DEPRESSION IN ORDER OF FREQUENCY OF THE ELECTRICAL COCHLEAR RESPONSE OF CATS[1]

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Since the discovery of the electrical response of the ear by Wever and Bray (1930), various experimenters have investigated the mechanism by which it arises in an effort to elucidate the mechanism of hearing. It has been shown that there are two electrical potential effects, one of which is obtained from the auditory nerve and the second from the cochlea, each having its own characteristics (Davis, Derbyshire and Saul, 1933; Davis and Saul, 1933).

Attempts to obtain light on the mechanism of the electrical response have involved the use of drugs (Adrian, Bronk and Phillips, 1934), and exposure to loud tone (Finch and Culler, 1934; Wever, Bray and Horton, 1934; Horton, 1934; Davis, Derbyshire, Kemp and Lurie, 1935). The response has been totally abolished by use of sodium chloride crystals placed on the membrane of the round window (Hallpike and Rawdon-Smith, 1934).

The last named investigators reported the elimination by sodium chloride of all frequencies. In connection with an experiment designed to see whether injection of quinine di-hydrochloride produced any loss in electrical response, the effect of sodium chloride crystals on the round window which was incidentally mentioned by the above authors, was corroborated. Furthermore, it was noted that the elimination of the cochlear response apparently took place earlier in the higher frequencies than in the lower.

In a preliminary paper we have recorded this differential elimination of frequencies (Fowler and Forbes, 1935). The present paper reports the detailed study with medium intensities of this progressive elimination from high frequencies to low by various agents and different concentrations as reflected in the electrical cochlear response of the cat. Corroboratory

[1] The study was made possible by grants from the Research Council of the American Otological Society and the Hayden Coakley Fund, the facilities of the Department of Pathology, of the College of Physicians and Surgeons of Columbia University and of the Department of Psychology of New York State Psychiatric Institute and Hospital.

histological evidence of progressive damage paralleling the electrical loss will be noted briefly and will be presented in detail elsewhere.

The evidence tends to support a "place theory" of hearing.

METHOD. *Apparatus.* The intensity of the electrical cochlear response was measured by recording the degree of amplification necessary to make the response just audible in the observer's head phones.

Two different capacitance-coupled amplifiers were used at different times. The first gave a maximum of approximately 80,000 times voltage amplification and the second approximately 100,000 times voltage amplification. Both amplifiers were designed to give a fairly flat amplification characteristic (±2 db.) from 20 to 15,000 cycles. Head phones were used.

The amplifiers were standardized with a test signal controlled by an attenuator calibrated in decibels. For a given amplifier setting, the signal was adjusted to threshold audibility with the identical phones and the

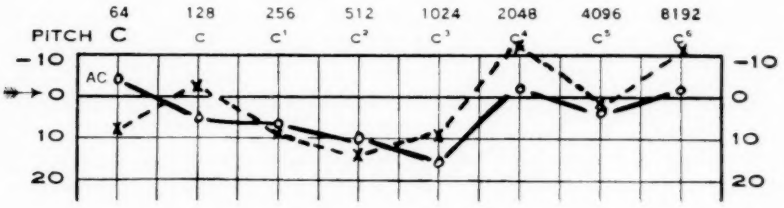

Fig. 1. Observer's audiogram

same observer as the experimental readings. In this fashion any idiosyncrasy of the amplifier-phone combination or of the observer's own audiogram was eliminated. A curve was run for each of the frequencies used. The observer's audiogram is given in figure 1 for completeness.

Electrodes were a zinc plated clip on exposed muscle (grounded) and a copper, sodium chloride active electrode placed on bone at the edge of the round window. This electrode had an insulating handle of glass tubing drawn to a fine tip and a gauze tip about 2 mm. in diameter to preserve moist contact and prevent damage. A shielded concentric electrode was tried but found unnecessary.

Fair sound proofing was obtained by placing the observer three rooms from the operating rooms and closing intervening doors and windows. Observer and operator communicated with each other by visual signals.

The auditory stimuli used were those readily available which were not possible sources of electrical artifact. Tones were obtained from an ordinary harmonica as low as approximately 256 while the higher tones were obtained from a Galton whistle set at readings of 10, 8, 5 and 3 which corresponded roughly to frequencies of 1000, 3000, 5000 and 10,000 re-

spectively. The observer adjusted the amplifier controls until the tone was just audible, and the amplifier settings thus obtained were translated into decibels of amplification from the appropriate standardization curve.

Agents were for the most part applied to the round window membrane either by dropping powder or a drop of the solution upon it. In some of the experiments the entire middle ear was filled with the experimental solution. In either case the ear was syringed out with normal saline and carefully sponged before each set of readings.

Operative exposure. Intraperitoneal pernoston anesthesia (approximately 0.6 cc. of 10 per cent solution per kgm.) was used. The operative exposure of the middle ear was for the most part through a diagnonal incision over the lower part of the parotid. With the animal's head on one side the parotid was exposed and its lower end dissected free and retracted. The bulla was then exposed by incision through the muscle directly over it, and a small window was cut in the bulla.

This method seemed superior to the technique which has been often used of exposing the bulla from a mid-line incision, since it occasioned the tying of no vessels. It also had the advantage of permitting a direct view of the round window membrane and the material placed in the niche of the round window remained there to better advantage when the cat's head was on one side.

For sterile operations and for checking the magnitude of electrical leakage from the opposite ear, the bullae were opened by the usual technique from a mid-line incision and two electrodes placed simultaneously in each side.

Histological method. The majority of the animals which were subjected to histological examination were perfused with normal saline followed by 10 per cent formalin, before removal of the temporal bones. After this they were further fixed with 10 per cent formalin. The usual hardening, decalcification and imbedding in celloidin followed. Every tenth section was stained.

RESULTS. Results are reported on a total of 31 cats, of which a preliminary series of 7 animals lacked a normal control ear. It was this series in which the characteristic high tone loss was originally noticed. The results are plotted in terms of the decibels of loss after the application of the experimental agent. The original set of readings after both ears had been exposed but before the agent had been applied, was used as zero loss.

Preliminary series. In the preliminary series intravenous quinine dihydrochloride produced no observable cochlear loss and no electrical response resembling tinnitus was noted. Sodium chloride crystals applied to the round window produced reduction of and elimination of the response, thus confirming Hallpike and Rawdon-Smith (1934). It was

further found that calcium chloride crystals and boric acid also eliminated the response as well as quinine when applied to the window membrane. The elimination was quite rapid, but it seemed that the loss appeared first in the high tones. Since both ears of the animal were used as experimental ears there was no control against possible artifacts from operative trauma, etc., and the results are considered merely exploratory.

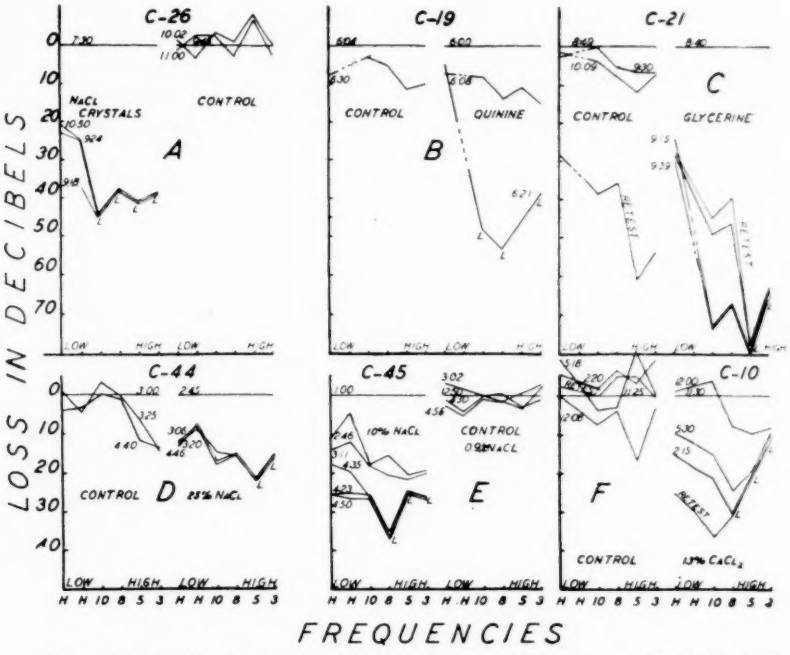

Fig. 2. Illustrating progressive differential loss. Frequencies = 256, 1000, 3000, 5000, and 10,000 respectively (H to setting 3). Left and right ear plotted on corresponding side of each diagram. *L* = limit of amplification reached. Agent indicated for experimental ear, control ear so marked.

Main series. In the remaining 24 animals the best ear was chosen for experimental purposes and the ear showing the poorer initial electrical response was operated and kept untreated as a control. All agents in the following sections were applied to the round window membrane.

Effective agents. Concentrated sodium chloride, quinine di-hydrochloride, glycerine, and calcium chloride produced a typical picture of cochlear loss which occurred first in the higher tones and later in the lower tones. Figure 2 shows the most typical records of such cases. Figure 2A, B, and C, indicate the effect of the concentrated agents. It will be noted

that in figure 2B and C, the loss is quite evidently progressive as frequency increases, while in figure 2A the increasing extent of loss with higher frequency is masked due to the fact that for the higher tones the limit of amplification is reached sooner than for the lower tones. This was due usually to a poorer initial response to higher tones. Older animals exhibited this to a greater extent than young ones.

With less concentrated agents, i.e., 25 per cent sodium chloride, 10 per cent sodium chloride and 13 per cent calcium chloride, the loss occurred more slowly and the loss in decibels showed a more nearly linear relationship with frequency (fig. 2D, E and F). As shown in figure 2E and F there was also fairly consistent increase of this loss with the time during which the agent was applied to the round window membrane, which loss

TABLE 1

Consistency of characteristic high tone loss

AGENTS	NUMBER OF ANIMALS		OBSERVATIONS ON ABERRANT ANIMALS
	Showing characteristic high tone loss	General loss	
Quinine di-hydrochloride	1	1	Ear full of blood muscular tremor
NaCl crystals	4, 3*†	2	No early reading. High tones poor originally
NaCl 10 and 25 per cent	3	1	High tones poor originally
$CaCl_2$, crystals and 13 per cent	1, 1*†		
Glycerine	2	1	

* From preliminary series.

† Corroborated by preliminary data on dogs.

did not change the general slope of the curve until the limit of recording was reached. Thus these two records allow us to follow the course of the process to better advantage.

Recovery. We obtained some evidence which indicated lack of recovery after from 1 to 25 days. In cat 21 (fig. 2C) there was a very extreme loss with glycerine at 9:39 whereas the control ear was comparatively unaffected as late as 10:09. On retest after 17 days this animal showed approximately the same loss in the experimental ear. Unfortunately at the time of retest the control ear was infected and also showed a gross loss, but in similar records in several other animals the control ear retained its sensitivity and the experimental ear its loss.

The variability of the control ear in figure 2F shows the extent of variation produced at times by none too favorable noise conditions in

the observer's vicinity. In spite of this variability it will be noted that the experimental ear shows two very similar curves at 2:15 and 5:30 and that the control ear shows no loss at 5:18.

Table 1 gives a summary of all the agents producing the characteristic progressive loss with increasing frequency. The table indicates the regular occurrence of the characteristic high tone loss with these agents. Although in 5 of the 24 animals a more general loss apparently occurred, in four of these trauma or other artifact made the record of questionable value.

Ineffective agents. Physiological sodium chloride solution and distilled water showed essentially no effect on the cochlear response. Figure 3A,

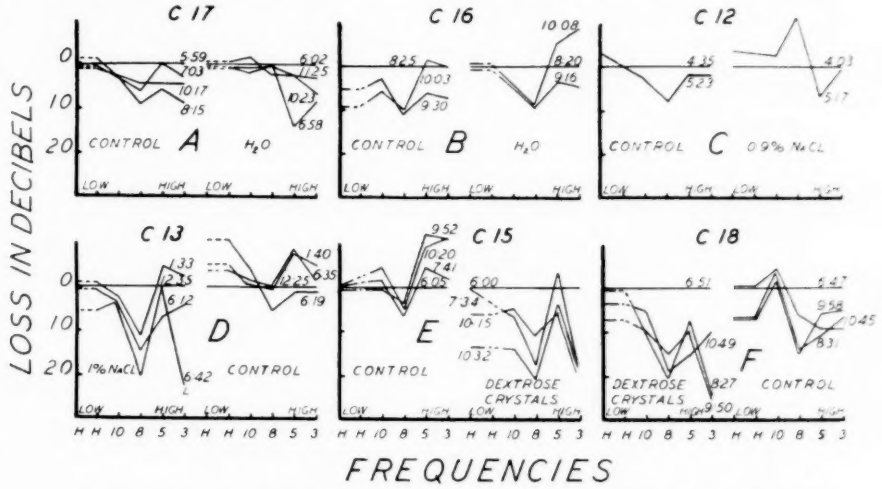

Fig. 3. Illustrating lack of differential loss. Plotting, frequencies and marking as in figure 2. Note that loss is in all cases slight, compared to that shown in figure 2.

B, C, and D, illustrate this fact. It will be noted that in none of the first three is there a loss greater than 10 db. due to either of the above agents and that this is offset by a similar loss in the control ear. The loss shown in figure 3D for one per cent sodium chloride is not consistent nor gross enough in our opinion to be significant for the same reasons.

Dextrose crystals produced a very questionable loss, as shown by figure 3E and 3F. Again the loss was general and not of a gross nature. It is barely possible that there is a slight loss in this case. Table 2 summarizes the results from agents giving essentially negative results.

Histological results. On microscopic examination of six of the experimental animals it was found that there was never any definite bulging of

either the round window or any of the membranes separating the scalae. There was often coagulated debris in the scala media and scala tympani of the experimental ears, but on the whole the main effect of the chemicals seemed to be confined to cells in the scala media. In the acute experiments where the temporal bones were fixed only a few hours after the application of the experimental chemicals, changes were apparent in the hair cells and in the large primitive cells of the external sulcus only. In one animal which was allowed to survive for twenty-five days there was degeneration near the apex of the cochlea in the outer hair cells and in the external cells alone (fig. 4A). Below this the inner hair cell and many more of the sustentacula cells appeared abnormal. Most basalward there was complete destruction of the sustentacula cells, the tunnel of Corti and also of the nerve fibers running in the spiral lamina (fig. 4B). The findings predicate that the electrical potentials of the auditory apparatus are initiated directly or indirectly by the hair cells of the organ of

TABLE 2

Consistency of negative results

AGENTS	NUMBER OF ANIMALS		OBSERVATIONS
	Negative	Inconclusive	
1.0 per cent NaCl	2		
H_2O	3		
Dextrose	2	1 (fig. 3F)	Some loss in control

Corti. We are unable to explain the early involvement of the external sulcus but feel that it is interesting in relation to the findings of Crowe, Guild and Polvogt (1934), in human high tone loss cases.

Discussion. *Possible artifacts.* The first and most obvious artifact which would produce a high tone loss of the type which we have found would be an increase in the shunt capacitance due to a change in concentration of the electrolyte either inside or outside of the round window. The latter was eliminated by careful sponging before each series of readings and at any time during the series when the intensity decreased. Furthermore, readings were taken with a 10,000 cycle note immediately following the first low frequency determination as well as in the reverse order so as to obtain the dryest surface during high frequency determinations. Since the reading from the control ears showed lack of high tone loss, such capacitative shunting across the outer surface of the cochlea (Girden, 1934) was evidently avoided.

In order to investigate possible internal electrolytic capacitance changes, a preparation was first tested for cochlear response and then rough meas-

Fig. 4. Illustrating cochlear destruction in a chronic preparation. Cat 28. Agent = NaCl crystals, applied 109 minutes. Animal allowed to survive 25 days, preparation perfused. All sections with 16 mm. objective, 8 × ocular.

A. *Degeneration of hair cells and sustentacula cells from apical turn.* Gray matter in media is hemorrhagic material. Arrows indicate degenerated areas. Dark material above left hand arrow is degenerated material.

B. *Complete degeneration from basal turn.* By comparing with the normal section it will be seen that not only the organ of Corti but also the sustentacula cells and those in the stria vascularis are destroyed.

C = *Normal section from control ear.* Section from basal turn corresponding to B.

urements were made of the two ears with an alternating current bridge at the various frequencies and with the same electrodes. In order to obtain the greatest possible difference between the ears, the loss in one ear was allowed to reach the recordable limit in all but the lowest frequency. As shown in table 3 there was a slight difference of capacitance and resistance between the ears but none great enough to account for the gross loss in the cochlear response.

Artifacts from the frequency characteristics of the amplifier or from the observer's audiogram were controlled by the method of calibration (see p. 25).

TABLE 3

Impedance of ears after gross loss in all frequencies. Cat 24, Nat'l crystals

	LEFT EAR (EXPERIMENTAL)			RIGHT EAR (CONTROL)		
Frequency	Bridge reading (parallel)		X_{cp}	Bridge reading		X_{cp}
	C_p (mF)	R_p	ohms	C_p	R_p	ohms
300	0 0674	1300	7875	0 1044	1172	5084
1000	0 0267	785	5964	0 0214	1010	7440
5000	0 0039	650	8165	0 0034	840	9366
10000	0 0019	610	8381	0 0018	735	8846

Decibels loss of cochlear potential response before and after bridge readings

	LEFT EAR								RIGHT EAR						
	Stimulus frequency								Stimulus frequency						
Time	300	1000	3000	5000	10,000	300		Time	300	1000	3000	5000	10,000	300
8:10	Agent applied														
9:27	41 5	34 5	24 5	9 0+	2 0+	40 0	(*Before*)	9:31	3 5	2 0	7 0	8 0	0	1 5
11:52	47 0	34 5+	20 0	9 5+	2 0+	33 5	(*After*)	11:48	1 0	6 0	7 5	4 5	0	−1 5

* The plus sign is used in the table of cochlear loss to indicate the attainment of the limit of amplification. The loss is therefore undoubtedly much greater.

Any effects due to anesthesia or to general traumatic injury, disturbances of circulation, etc., should be reflected in the parallel readings from the opposite untreated ear.

Partial ankylosis of the ossicles by blood clot, or edema of the membrane in the niche of the round window, should cause the opposite effect, i.e., greater loss in *low* tones.

Type of destruction. In the animals so far studied the experiments indicate that the lesions produced by electrolytes on the round window are similar and perhaps identical with the lesions of so-called senile deafness, drug poisoning and the deafnesses produced by the exposure of the

ear to long continued, very loud tones, since similar pathological findings are present.

Evidence for localization of tones. On the basis of our present evidence and within the limitations of accuracy of our set-up, we believe that the high tone loss is an evidence for localization of the high tones at the basal end of the cochlea and of the lower tones progressively toward the apex. This follows from the fact that the agent perfusing through the window membrane would be more concentrated initially in that region and that the concentration change in the cochlear fluid would be propagated slowly toward the apex. The differential loss thus is indicative of localization similar to that postulated in "place" theories and is in line with reports suggesting such localization with other techniques (Crowe, Guild, and Polvogt, 1934; Hallpike and Rawdon-Smith, 1934; Davis, Lurie and Stevens, 1935; Culler, 1935).

The mechanism of the loss. The loss produced might be supposed to be a matter of osmotic pressure differences, since concentrated solutions and glycerine produced the characteristic loss. However, if such is the case, it is necessary to explain the fact that pure water and dextrose produced practically no loss. Furthermore, there is no evidence from the histological preparations of a maintained pressure difference either in respect to the membranes dividing the scala or in the round window itself.

At present the best tentative hazard at the mechanism producing the loss seems to be that the agents produced a change of permeability of hair cell membranes and that this interfered with the setting up of a chemical concentration difference between the cell interior and scala media, or with release of ions so accumulated. Either a chemical mediator, such as that postulated by Derbyshire and Davis (1935), or cell membrane depolarization effects would be thus interfered with.

It has been shown that an *increase* of permeability of cell membranes of simple forms is produced by supernormal NaCl and $CaCl_2$ and in the extreme case is accompanied by death of the cell. Differential concentrations of K and Na are necessary to life in these forms. Mechanical stimuli result in permeability changes and exudation of sap from the cell interior (Osterhout, 1922, 1935). It is suggested that an analogous situation in hair cells and sustentacular cells would explain the production in stimulation deafness of destruction like that in our specimens, and reduction of response with the agents used.

Decrease of permeability was obtained by Osterhout with certain agents. We have been unable to show a reversal of sensitivity with $CaCl_2$ similar to Osterhout's in permeability but this may be due to the roughness of our measure. It is tentatively suggested that the increased permeability effect above noted gives the better explanation of our results.

The source of the cochlear potentials. The occurrence of destruction of

outer hair cells alone with loss in amplitude of cochlear potentials indicates the hair cells to be sources of such potentials.

Apparently both cochlear and nerve processes were actually affected by our agents, since it has been reported that 25 per cent of the amplitude recorded at the round window represents nerve action currents (Derbyshire and Davis, 1935), and it is otherwise impossible to account for so great a loss in our records. If so, the hair cells are apparently direct or indirect initiators also of auditory nerve stimulation. Our results thus support the postulation of numerous investigators. The hair cells are apparently necessary for maximum sensitivity (cochlear potential response) to medium intensities.

A study is now in progress using audiograms of dogs taken by means of a conditioned response technique (Culler, Finch and Girden, 1933), to investigate the effect on hearing.

We are greatly indebted to Mr. Wm. McKnight for aid in preparing the data.

SUMMARY

1. The effect of certain agents on the electrical cochlear response of cats has been investigated by means of the action current technique. The intensity of potentials from the border of the round window, was measured by determining the amplification necessary to make them just audible.

2. Quinine di-hydrochloride, sodium chloride and calcium chloride crystals, sodium chloride and calcium chloride solutions of from 10 to 25 per cent and pure glycerine when applied to the round window produced a characteristic picture in which a gross loss of electrical cochlear response occurred earlier in higher tones than in lower tones. The degree of loss increased with the time that the agents were allowed to remain on the round window. Sections of the cochleae of cats so treated showed various degrees of cochlear degeneration occurring first at the basal end and finally in the apical turn of the cochlea. The extent of the degeneration depended upon the amount of time during which the agents were applied and the time which elapsed between the application of the agents and sacrifice of the animals.

3. Physiological sodium chloride and pure water produced no apparent loss. Similarly dextrose produced a very questionable loss.

4. The characteristic high tone loss is interpreted as evidence for localization along the cochlear spiral of end organs which record tones of medium intensity. Such localization is apparently in order of frequency with the high tones at the basal turn as postulated by Helmholtz, and lower tones progressively toward the apex. Our findings are thus in agreement with those of other investigators using other techniques.

5. In the light of evidence for a chemical mediator from other studies

our results are tentatively interpreted as due to permeability changes which interfere with the formation of or which reduce the liberation of a chemical mediator from the sensory cells. Cell membrane potentials would also be involved.

6. The histologic data indicate that the hair cells are indispensable for maximum sensitivity of cochlear potential response to tones of medium intensity and are primary in the normal activation of the nerve.

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A STUDY OF THE AVERAGE TEMPERATURE OF THE TISSUES, OF THE EXCHANGES OF HEAT AND VASOMOTOR RESPONSES IN MAN BY MEANS OF A BATH CALORIMETER

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Some of the earliest measurements in animal and human calorimetry were made by observation of the heat given to water baths in which the body was immersed. The great pioneer in the field was Liebermeister (1875) and his pupil Kernig. Considerable improvements in the method were made by Lefèvre (1911) who discussed at length the accuracy and usefulness of the method. Modern human calorimeters make use of an air chamber so that the heat exchanges of the body with its surroundings are those of normal physiology. Since the whole body cannot conveniently be immersed, the usefulness of the water bath as a calorimeter to measure the total heat lost by the body is obviously limited, but for some purposes the water bath possesses advantages over the air chamber, namely, in the study of the mechanism of heat loss by the immersed portion of the body with the surrounding medium.

These advantages arise from the greater efficiency with which heat is exchanged in well stirred water than in air, so that the temperature of the surrounding medium is kept very closely uniform throughout. In addition, the temperature of the surface of the body cannot be more than a fraction of a degree different from that of the water of the bath and may be made to change in any desired manner by changing the temperature of the water. In air the temperature of the surface of the body varies physiologically over a wide range and it is by no means easy to measure the average surface temperature over a large area of the body. It should be remembered, however, that the production of a uniform surface temperature creates a somewhat abnormal physiological condition, which may be associated with abnormality of reflex responses. As it was desired to study how well, or how badly, the changes in deep rectal temperature of the body could serve as an indication of changes in average temperature of the tissues, factors could be more readily controlled if the surface temperature was kept constant while the rectal temperature was changed, and vice versa. Vasomotor reactions to temperature may arise from either

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direct effects of the blood temperature upon the heat regulating center or from sensory impulses from the periphery. If the surface temperature may be controlled by the bath temperature, it is possible to study separately the rôle of these two reflex mechanisms. In the interpretation of heat loss from the surface or in the use of surface temperature as an indication of peripheral blood flow, the evaporation of water from the skin is a complicating factor (Burton, 1934a). From the immersed surface no heat is lost by evaporation, even though loss of water through the skin may continue, and the interpretation of thermal changes is correspondingly more direct.

These reasons led to the adaptation of a bath, used previously in studies of circulatory and respiratory responses to temperature (Bazett, 1924) to measure the heat exchanges of the immersed body with the water.

METHOD AND ITS STANDARDIZATION. The bath is an ordinary household bathtub equipped with a stirring device which draws water through a rubber tube from the "head" end and discharges it at the foot of the bath. The temperature of the bath is controlled by a toluol-mercury regulator which runs the length of the bath down one side. This actuates relays which pass or shut off current from the 110 volts A.C. line through three metal immersion heaters (G.E. Hotpoint—500 watts each), mounted near the stirrer at the foot of the bath; in calorimetric periods one of these suffices to keep the bath at constant temperature. The fluctuations of a mercury-in-glass thermometer at the other side of the bath show that the regulator keeps the temperature of the water at this point constant, within two or three hundredths of a degree centrigrade. The level of the water in the bath is kept constant by an overflow device set to a definite level. During the experiments, if it is necessary to remove water, it is siphoned out and may be weighed, so that the volume of water displaced by the subject is known.

The deep rectal temperature of the subject is continuously recorded by means of an electrical resistance thermometer and recording bridge (Leeds and Northrup) accurate to about 0.04°F.

The calorimetric device is a simple addition to the bath. The closing of the contacts of the relay that turns on the heaters also passes current through a self-starting electric clock (Telechron), which thus records the total time that the heater has been on. In a parallel circuit is also a similar clock motor which, during the time in which current passes, turns a small pulley on its shaft at the constant rate of one revolution per minute. A thread passes round this pulley and lowers steadily, while the heater is on, a stylus which marks a sloping line on the moving smoked paper of a kymograph. When the regulator turns off the heater, the clock motor stops and the stylus marks a horizontal line on the record. There is thus a record not only of the total time the heater has been on (by the Telechron

clock) but of the moments at which it was turned on and off by the thermal regulator of the bath. Knowledge of the resistance of the heater units and of the average voltage applied to them (this is recorded during the experiments) enables one to calculate the number of kilocalories supplied by the heater in any given period of elapsed time.

The principle of the measurement of the heat given up by the subject is simple. To maintain the temperature of the bath at a constant level, the heater must supply heat at a rate determined by the difference between the temperature of the water and that of the room. The humidity of the room also may affect the heat loss, but this is minimized in the experiments. When the subject is in the bath, heat is given to the bath from the body, and, consequently, the electric heater is turned on less often by the thermal regulator to make the total heat supplied equal to the heat loss of the bath. Comparison between records taken with the subject in the bath, and control experiments with no subject in the bath, after correction has been made for variation in the heat loss of the bath due to any fluctuations of room conditions, gives the heat supplied by the subject.

In order that the contribution of heat by the subject might be a large proportion of the total heat required to balance the heat loss of the bath, the latter was reduced by the enclosure of the bath by a wooden frame containing a layer, about one inch in thickness, of "Rockwool." About two-thirds of the surface of the bath is covered with a permanent layer of paraffin wax, about one inch in thickness, supported by suitably placed struts. When the subject has entered the bath the remaining surface is covered by a removable section of wax, leaving a small opening for the neck of the subject. This opening is finally closed by a collar of rubber, worn by the subject and sealed to the wax by pouring on a little of the melted wax.

The result is that evaporation from the bath is made very small so that the heat loss of the bath is insignificantly altered by the changes in the humidity of the room that might occur between calorimetric and control periods. With the water temperature in the neighborhood of 35°C. and the room at 25°C. the loss of heat by the bath is of the order of 2.5 kilocalories per minute. Since an adult subject produces and eliminates something like 1 kilocalorie per minute, the change to be expected in the heat supplied by the heater is a large percentage of that supplied in the control period. In fact, if the bath temperature is below 30 or 31°C., the heat given up by the subject, unaided by the heater, may be sufficient to keep constant or gradually raise the water temperature. In such cases the calculation of the amount of heat supplied is possible from a knowledge of the thermal capacity of the bath and its rate of rise of temperature. This measurement is, of course, much less accurate than the first method, due to the large thermal capacity and impossibility of measuring very small

changes of temperature of the water; it was only necessary to resort to it in a few cases during the experiments. Incidentally, it is the method that was used by the older investigators.

Heat production was calculated from the oxygen consumption, measured in a Sanborn, using the factor of 4.8 kilocalories per liter of oxygen.

Control experiments. A series of control experiments was made with the bath temperature regulated at temperatures from 30°C. to 38°C. to find how the heat loss of the bath was related to the temperature of the water and that of the room. Since the amount of circulation of the air affects greatly the heat loss, it was decided throughout the experiments to maintain a steady state of circulation by a fan rather than to rely upon the constancy of unstirred room air. A linear relation was found between the rate of heat loss and the "excess temperature" of the water over that of the room (dry bulb temperature). The humidity of the room made no significant difference to this relation. The following procedure was therefore justified. After a series of periods with the subject in the bath were completed (lasting usually a total of four hours), the bath was resealed with wax in as close an imitation to the original seal as possible and two control periods were run, usually each of one hour duration, at two bath temperatures such that the corresponding excess temperatures included between them any excess temperatures occurring in the experiment. The heat loss in any periods could then be calculated by interpolation. An example will make the procedure clearer.

1. Period with subject in the bath.

	Actual elapsed times	*Reading of heater clock*
	A.M. 11:56 :00	22′:12″
	11:34 :20	18′:28″
Subtracting	21′:40″	3′:44″

The heater was "on," on the average, $\frac{224}{21.67} = 10.33$ seconds per minute of elapsed time. Average voltage on heater = 112.8. Bath temperature 33.57°C. Average room temperature 24.03. Excess temperature = 9.54°C. Then Cals./Min. supplied by heater = $(112.8)^2 \times 0.009105 \times 10.33 = 1.20$ Cals./Min. (0.009105 is a factor involving the resistance of the heater.

2. Control periods. Similarly it was found, after the subject had left the bath

Heater supplied 2.781 Cals./Min. with excess temperature 12.04°C.

Heater supplied 1.501 Cals./Min. with excess temperature 8.02°C.

Correction Factor 1.28 Cals./Min. for 4.02°C. = 0.318 Cals./Min./°C.

In the experimental period (1) above, the excess temperature was 9.54°C. The heat loss in that period was therefore:

$1.501 + (9.54 - 8.02) \times 0.318 =$	1.99 Cals./Min.
Of this the heater supplied	1.20 Cals./Min.
Therefore the subject supplied	0.79 Cals./Min.

Thermal capacity of the bath. This was found experimentally by measurement of the number of calories supplied by the heaters in raising the bath from constancy at one temperature to final constancy at a higher temperature, making allowance for the heat lost to the room in the period of transition. The result was 284 ± 4 kilocalories

per degree C. for the effective thermal capacity. As the bath contains 280 liters of water when filled to the constant level, a result of this magnitude was to be expected. For periods with the subject in the bath where the thermal capacity had to be used, a number of calories equal to the liters of water displaced by the subject was subtracted.

Accuracy and thermal lag of the heat measurement. To test the accuracy of the measurements under the conditions in which they were to be made, heat checks were made with a 100 Watt lamp (producing 1.23 Cals./Min.) immersed and sealed into the bath in place of the subject. The changes that occurred in the type of record when the lamp was turned on are illustrated by figure 1. The heater, which has been "off" for, on the average, 1.7 minute and "on" 0.8 minute alternately, changed very rapidly when the lamp was turned on to a routine of "off" about 5.5 minutes and "on" for 0.6 minute. These changes show the decreased rate of cooling of the bath and its increased rate of rise of temperature when the lamp was "on." Five minutes were adequate to cover the period of thermal lag. The simultaneous values of the current through the lamp and the voltage across it were measured, so that the number of calories produced per minute was known. The heat received by the water was calculated from the records exactly as with the subject in the bath. Using twenty minute periods the two quantities agreed within 4 per cent. This accuracy is considered very satisfactory for so simple a calorimetric device using periods of such short duration.

Fig. 1. Record of the time the heater was on and off in a control experiment in which an immersed lamp was turned on at point marked with an arrow. Abscissae: time in minutes. A horizontal line indicates that the heat was off.

Surface temperature. It is fundamental to the interpretation of the results that the temperature of the immersed surface of the body should differ from the temperature of the water only by a negligibly small quantity. The great ease with which an immersed body exchanges heat with well stirred water suggests that this is true, but the point was experimentally proved. As the superficial tissues are relatively poor conductors of heat, it is difficult to be sure that a thermocouple passing through the water to the skin might not produce a local cooling of the point of the body surface with which it was in contact, and so give an erroneous idea of the surface temperature. Instead of the subject, therefore, a hollow cylindrical copper vessel, of length 51 cm. and diameter 16 cm., was immersed in the position of the trunk of the subject in the bath. The great thermal conductivity of copper prevented any possible local cooling by the thermocouple wires. An electric lamp inside the cylinder carried sufficient current to generate heat which was eliminated from the cylinder's surface to the water at a rate equal to 36 calories per square meter/hour. A differential thermocouple recorded the difference of temperature between the surface of the cylinder and the point where the bath thermometer was placed.

It was found that while in unstirred water there was established a temperature difference between these two points greater than 1°C., yet, when the stirrer was operating, the temperature difference was less than 0.08°C. (the smallest measurable temperature difference). The temperature of the immersed surface of the body may then be taken, without appreciable error, to be equal to that of the bath water in the experiments.

EXPERIMENTAL MATERIAL. The series of experiments here reported consist of 10 bath experiments upon one male subject (HCB), using nine-

teen different bath temperatures and comprising in all 85 periods for which the heat exchange was calculated. One experiment was made with each of two other male subjects (H and G), three experiments on a female subject (M) and one on a second female subject (R). It is obviously not expedient to give the details of this number of experiments. The routine and details of two of the experiments which may be taken as typical are, therefore, given, followed by a brief statement of other experiments. The results obtained are stated in a more general form in the discussion.

Experiment 1. The results of this experiment are shown in figure 2. The subject, in basal condition, entered the bath at 9:40 a.m. The volume of water displaced was measured (60.1 liters), the rectal thermometer adjusted by the subject, and sealing

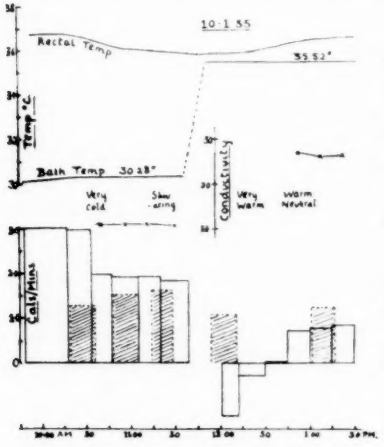

Fig. 2

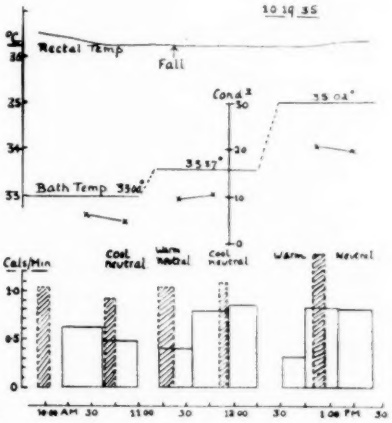

Fig. 3

Fig. 2. Experiment 1. Abscissae: time in minutes; ordinates: temperature of bath and rectum; index of conductivity and calories per minute. The heat given to the bath is indicated by open blocks; that produced, by shaded blocks.

Fig. 3. Experiment 2. Indications as in figure 1.

by wax completed. To reduce the heat loss from the head a rubber bathing cap was worn with a rubber "veil" over the eyes and nose. A preliminary period of ten minutes was necessary to attain steady conditions. In the next thirty minutes the bath temperature rose without any heat being supplied by the heater, as the result of the heat given up by the body in the cooling of the peripheral tissues. The bath regulator was re-adjusted at 30.28°. From this point the bath temperature remained constant; the regulator turned the heater on with increasing frequency as the heat from the body decreased. The rates at which heat was given to the water by the body are shown by the blocks in the figure (full lines), and also in table 1. After the first two periods, a steady state was reached after which the heat given to the bath decreased very little, and this decrease was parallel to the decrease in the difference of temperature between rectal temperature and bath temperature (Δ T in the table). Even without the heat loss from the head, the heat given up by the body to the bath

exceeded the heat production—shown in the diagram by the shaded blocks. The metabolism increased throughout this part of the experiment, while the subject felt cold and eventually shivered.

At 11:40 a.m., the bath temperature was raised in the space of 11 minutes to 35.52°C. by syphoning out some of the cold water and replacing it by hot water from the tap, the level being maintained constant throughout.

As table 1 and the figures show, for the first thirty minutes the heat given up by the subject to the bath was negative, that is, not only was all the metabolic heat conserved by the body, but heat was accepted from the bath water. As will be amplified later in the paper, this could only be the case if the temperature gradients in the peripheral tissues were reversed. The heat exchange finally reached a positive comparatively steady level. Again in the steady state, the heat given up closely paralleled the difference of temperature between rectum and bath. The last column of table 1 is obtained by dividing the heat given to the bath by the subject, expressed

TABLE 1

10:2:35. Subject, HCB. Weight, 67.3 kgm. Entered bath 9:40 a.m.

TIME	RECTAL TEMPERATURE	BATH TEMPERATURE	HEAT TO BATH	HEAT PRODUCED	Δ T	$\frac{H}{\Delta T}$
a.m.			*cals./min.*	*cals./min.*		*cals./sq.m./hr./°C.*
9:50 to 10:18	36.66 to 36.63	30.06	+3.02		6.59	
10:18 to 10:32	36.63 to 36.43	30.22	+2.99	1.29	6.31	
10:32 to 10:47	36.43 to 36.17	30.27	+1.99		6.03	11.0
10:47 to 11:05	36.17 to 36.11	30.28	+1.92	1.53	5.86	11.0
11:05 to 11:20	36.11 to 36.00	30.28	+1.93		5.78	11.0
11:20 to 11:39	36.00 to 35.93	30.29	+1.85	1.63	5.69	10.8
p.m.						
12:01 to 12:12	35.89 to 35.93	35.52	−1.20	1.08	0.39	
12:12 to 12:30	35.93 to 36.14	35.52	−0.18		0.53	
12:30 to 12:45	36.14 to 36.33	35.52	+0.04		0.72	
12:95 to 1:00	36.33 to 36.47	35.52	+0.71		0.88	27.0
1:00 to 1:15	36.47 to 36.56	35.52	+0.79	1.21	1.01	26.0
1:15 to 1:30	36.56 to 36.65	35.52	+0.87		1.09	26.7

in calories per hour per square meter of body surface immersed, by the difference Δ T. Its constancy in the steady state shows that these two quantities are proportional. It will be seen, however, that the value of this constant is greatly increased in the hot bath over that in the cold bath, since the heat has decreased much less than the temperature gradient across which it is flowing. As will be shown later, this indicates an increase in the effective thermal conductivity of the peripheral tissues. The rectal temperature rose, after a lag period, to a comparatively steady temperature.

This experiment illustrates those in the series in which the changes in the bath temperature, and therefore surface temperature, were, physiologically speaking, violent, from a cold bath, where "chemical regulation" by increased metabolism is called upon, to a very warm one where physical regulation by increased peripheral blood flow is inadequate to keep the body temperature from increasing steadily. Presumably, its level would

ultimately rise high enough above the bath temperature to achieve equilibrium. Characteristics to be seen are the large amounts of heat involved in the cooling and heating of the peripheral tissues, the delayed responses of the rectal temperature, and the period of reversed peripheral gradients.

Experiment 2. This is illustrated by figure 2. Here the negative heat exchange indicating reversed peripheral gradients is not evident, though it might occur for a brief period and yet not appear in the calorimetry taken over a longer period. The changes in the effective thermal conductivity are such that when the steady state is reached at the new bath temperature, the heat given up is at approximately the original level. In the case of the first rise of bath temperature, the heat given up to the warmer bath was finally greater than that originally exchanged in the colder bath. Here then the vascular response has over-compensated for the decrease in

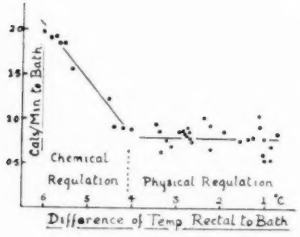

Fig. 4

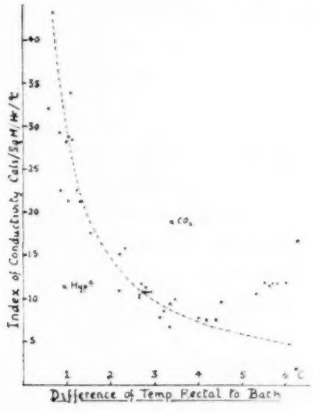

Fig. 5

Fig. 4. Heat given to bath relative to Δ T in subject HCB.

Fig. 5. Index of conductivity relative to Δ T for subject HCB (summer).

The curve drawn is a theoretical hyperbola, from which the data diverge at high temperature differences.

the difference of temperature (Δ T) between rectal and bath temperatures. In the case of the second rise of bath temperature, the adjustment is quite accurate, the final level of heat exchanged being very close to the original.

In this experiment, which is typical of those where the changes in bath temperature were more moderate, a new phenomenon appears—namely, that an increase in the bath temperature produces an initial fall of rectal temperature, although the calorimetry shows that the body has a positive heat balance and, therefore, that the true average body temperature is rising.

Other subjects gave similar results but showed some individual differences, part of which may have been due to seasonal changes, since, as will

be seen later, even the single subject showed some difference at different times of the year. The area not immersed in the water is a source of variation, and forms a larger proportion of the total surface in the smaller subjects; it amounted, for instance, to 7.8 per cent of a total of 1.8 sq. M. in HCB, while in the smallest subject, M, it was 8.9 per cent of a total of 1.35 sq. M.

RESULTS AND DISCUSSION. *Limits of physical regulation.* The data obtained in the summer months on subject HCB are plotted in figure 4. The ordinates are the heat given to the bath by the subject when the steady state was reached in each instance. With deep to surface temperature differences of 4°C. or less the heat exchange is constant. Over this range the variation to be expected from the changes in the temperature gradients between warmer and colder baths is compensated by physiological adjustments in the effective thermal conductivity of the tissues. With greater temperature differences the compensation is incomplete, heat loss is increased and increased heat production may or may not suffice to prevent lowering of the body temperature.

Effective thermal conductivity index as a measure of peripheral circulation. The constancy of the temperature of a given volume of deep tissues requires a balance between the heat production in that volume and the heat leaving it across its boundaries. The latter follows the fundamental law of heat flow, namely:

$$\text{Flow of heat} = \text{Effective Thermal Conductivity} \times \text{Cross-sectional area} \times \text{Temperature Gradient}$$

(The term "effective thermal conductivity" is not to be thought of as implying "conductivity" in the strict technical sense, but as an index of the ease with which heat flows through tissues by the combined means of direct conduction and "convective" transport of heat by blood flow.) Reflex control of the peripheral blood flow can so alter this index in response to changes of bath temperature, and consequently of gradients, that over the range shown in figure 4 the flow may be kept constant. The vascular response may then be quantitatively expressed in terms of thermal conductivity by transposition of the fundamental equation to the form:

$$\text{Effective Thermal Conductivity} = \text{Heat flow per unit area} / \text{Temperature Gradient}$$

The heat flowing through unit area of the immersed surface to the water is measured in the experiments. The appropriate gradient, if an average value for the thermal conductivity is required, would be the average gradient in the tissues. It can be shown, quite generally, that if two steady states be compared, where the gradients have the same relative distribution with depth in the body, this average gradient is proportional to the difference of temperature between any two chosen depths. Thus a number pro-

portional to the effective thermal conductivity is obtained by dividing the heat given to the bath per unit area by the rectal-surface temperature difference (Δ T of table 1). To obtain the value in the usual units of thermal conductivity this ratio would have to be multiplied by the effective thickness of the tissues through which the heat had flowed.

The values of the effective thermal conductivity for all the summer experiments on subject HCB are shown in figure 5. Only data appropriate to steady states rather than to periods of transition in which gradients were abnormal are used. The dotted curve of figure 5 is a theoretical hyperbola, such that the heat loss (conductivity $\times$ gradient) to the bath would remain constant at 30 kilocalories/sq. M./Hr. Over the range of

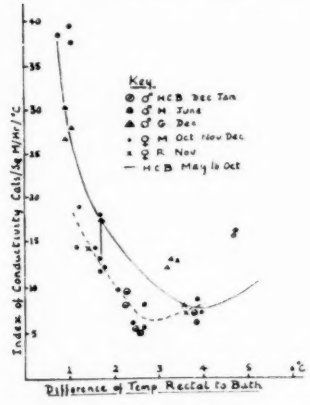

Fig. 6

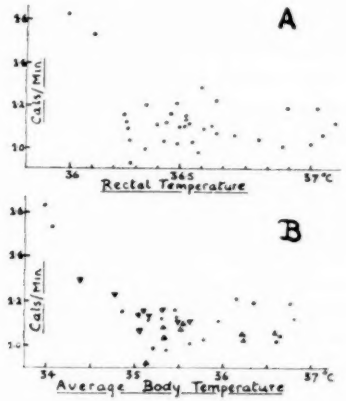

Fig. 7

Fig. 6. Comparison of conductivity indices for other subjects and for winter data on HCB with summer curve for HCB.

Fig. 7. A. Relation of heat production to rectal temperature in subject HCB.
B. Relation of heat production to average body temperature in subject HCB.

bath temperatures from 1° to 4° below rectal temperature the points lie close to this line.

In colder baths the effective conductivity after reaching a minimum begins to rise. The minimum coincides with the point at which metabolism begins to increase, eventually leading to shivering. The explanation may then be found in the increased circulation that must accompany increased activity of the peripheral muscles, which would inevitably entail an increase in effective conductivity. Since the blood vessels to the skin pass through the muscles, when cutaneous vasoconstriction is maximal any heating of the blood in its course through muscle must lead to increased heat loss. Thus the method of maintaining body temperature against cold by increased heat production is an inefficient one. Not only has

more energy to be consumed, but in its consumption heat loss is made greater than it would otherwise be. The indices for other subjects and some obtained on subject HCB in the winter are shown in figure 6 for comparison with the summer data on HCB (shown by the full line curve). The data obtained in the winter on subject M, R, and HCB appear to follow a lower curve with a different minimum (shown in the dotted line). On the other hand, one male subject in winter gave data falling on HCB's summer curve. Individual differences undoubtedly exist, but there is evidence also of a seasonal variation. The values for HCB in winter lie significantly below the minimum values obtained in summer. A seasonal variation might be anticipated in view of the known effect of environmental temperature on blood volume, for Barcroft et al. (1922) found increases of 700 cc. to over a litre as a result of exposures to temperatures not significantly different from some common in Philadelphia in the summer. Full vascular dilatation or constriction in the skin as a whole may be difficult to attain without alteration in blood volume, and the slow development of full adjustment to changes in environmental temperature that is common experience may depend in part on an alteration in blood volume.

It was thought that the temperature of the room, which varied between 21° and 27°C., might play a part in determining the vasomotor response to the temperature of the bath, but variations in the average temperature of the face did not produce significant changes in conductivity. With subject M., periods in a bath at constant temperature were compared in which first an electric radiation heater and second a fan were directed on the face. Variations in the average temperature of the face, measured by a light thermocouple, were from a normal of 32.5° to 37.4° and 30.3°. The only significant change of conductivity was produced by the heater, and this effect is shown in figure 6 by the arrow. Variations in room temperature must have acted as stimuli of much lower order.

Figure 5 shows that the effective thermal conductivity of the tissues of HCB was changed by vascular response by a factor of about six times. A lower limit of the conductivity below which that of the tissues could not conceivably fall would be that found for tissues in which there was no blood flow at all. Thermal conductivities of tissues removed from the body were compared with those of an equal thickness of air by Bordier (1898). His results would imply an average figure of about 0.0002 unit (cals./sec./sq. cm./unit temperature gradient), while assuming an "effective thickness" of the tissues of reasonable magnitude, the minimum value obtained in these experiments was of the order of 0.001. An upper limit to the conductivity obviously exists, determined by the maximum blood flow that may be diverted to the periphery of the body. Suppose that to keep in thermal equilibrium 40 calories per sq. M. of body surface per hour must be carried to the surface. In a bath that was one degree cooler than the internal blood temperature, the blood in passing from the heart to the

surface could cool by not more than this one degree. To transfer the heat, a circulation of 40 liters/sq. M./Hour would be the minimum requirement, which is one-third of the total basal cardiac output. The limit to the proportion of the total output that can be diverted to the periphery means that thermal equilibrium cannot readily be attained in a bath less than one degree or so lower in temperature than the body.

Reflex origin of the response. The immediate response to temperature would appear to be mainly reflex in origin, in view of the absence of a relation to changes in rectal temperature. For instance, an increased conductivity accompanied decreased rectal temperature when a cold was followed by a warm bath. Sensations usually showed a similar lack of correlation with the level of rectal temperature and its direction of change. The deep body temperature level or its direction of change can, however, play a part. This was demonstrated in an experiment in which a subject exposed to a bath of 33.7° for over an hour had apparently achieved thermal equilibrium and moderate comfort. At this time a dose of one drop of 1 per cent nitro-glycerine in alcohol was given under the tongue. There resulted a sharp fall of rectal temperature (0.2°C.) within two minutes and this was accompanied by temporary intense shivering and increased oxygen consumption. The heat exchange to the bath was increased, though this was not seen until later. Shivering ceased while the rectal temperature remained at its lowest level (37.2°C.) presumably because it had ceased to fall or because of some modification of thermal gradients in the region of the receptors. Changes of reflex origin, however, were more prevalent in the experiments, and it may be pointed out that since heat flow is the product of conductivity and thermal gradient, it could theoretically be best controlled if thermal conductivity were regulated by the value of the gradient.

Sensitivity of the method for measuring other circulatory changes. The effect of a small dose of nitroglycerine has been mentioned. Doses of this order were used twice and in each case gave an increase in the thermal conductivity index of about 20 per cent, which reached its maximum in 15 minutes. The pulse rate changes reached their maximum in 4 minutes and had subsided in 8 minutes. The delay in the thermal exchange is attributable to the lag in the temperature changes of the peripheral tissues. Changes in the CO_2 tension of the blood may also produce considerable effects. In one experiment with a bath temperature of 36.3° the subject went into voluntary hyperpnea for 2 minutes; in another, with the bath at 33.0°, he breathed a 10 per cent CO_2, 90 per cent O_2 mixture for 3 minutes. The results are shown in figure 5 and table 2. With hyperpnea there resulted a sharp rise, with CO_2 a marked fall of rectal temperature, though the degree of muscular respiratory activity was similar; presumably the rectal temperature changes resulted from vasoconstriction and vasodilatation in the skin.

The work of Liljestrand and Magnus (1922) indicated that marked changes in skin circulation might result from baths containing CO_2 and the question arose whether the retention of CO_2 by the paraffin wax cover might modify the skin circulation.

Analysis of the water by a Van Slyke apparatus showed the CO_2 content of the water to be increased to a barely measurable extent, even after the subject had been in the water for several hours. On the other hand, increase in the CO_2 of the water to about 180 mm. produced by bubbling CO_2 through it, or complete removal of CO_2 by the addition of an excess of baryta water produced barely measurable changes (less than 8 per cent) in conductivity and only fleeting sensations of temperature. Accumulation of CO_2 in the water was, therefore, inadequate to produce measurable changes.

Average body temperature. Though the effective thermal conductivity cannot be estimated except in a state that is approximately steady, the initial thermal exchanges between the body and the bath may be measured, and the change in average body temperature be determined. The main sources of error depend on the specific heat capacity assigned to the body, and to the fact that the initial exchanges in the first few minutes, before the bath temperature itself has become steady, cannot be measured. The measurements of heat exchange demonstrate that the superficial tissues

TABLE 2

PERIOD	DURATION OF CALORIMETRIC PERIOD	HEAT GIVEN TO BATH	INDEX OF CONDUCTIVITY	AVERAGE INCREASE
	minutes	*cals./minute*		*per cent*
Control	15	0.54	22.4	
Hyperpnea	13	0.31	11.9	−46
Control	15	0.62	21.3	
Control	21	0.73	7.6	
10 per cent CO_2	18	1.42	15.2	+100

have considerable changes in temperature extending to considerable depths, so that the heat loss or acceptance is large. Though the heat loss from the lungs and head is not measured it may be estimated from the discrepancy between heat production and heat loss in the steady state. If this heat loss from the head, which is relatively small (e.g., 0.25 kilocalorie/min. in subject HCB), be considered constant, the total heat absorbed by the body between steady states at two different bath temperatures may be calculated (e.g., between 11:40 and 12:45 in fig. 2) and the change in average temperature be calculated, since: (heat absorbed) = (weight × (specific heat capacity) × (rise of average body temp.). Such estimates may be compared with the heat exchange calculated on the assumption that the change in rectal temperature indicates the change in average body temperature, the usual procedure in calorimetry. The results are shown in table 3. Previous work with Prof. J. R. Murlin by one of us (Burton 1934b) has shown that better agreement is obtained between direct and indirect calorimetry, when a formula, which combines rectal and surface

temperatures, is used to estimate average body temperature. This formula is:

(Average body temp.) = 0.65 (Rectal temp.) + 0.35 (Surface temp.)

The heat so calculated is also shown in table 3.

When the surface changes by large amounts, more than 3°C., the rectal temperature is quite inadequate for the calculation of the heat absorbed,

TABLE 3

Changes in average temperature

OBSERVED CHANGES			HEAT ABSORBED OR LOST				
Duration of period	Rise of rectal temperature	Rise of surface temperature	Measured	Calculated, rectal	Error	Calculated Formula	Error
			Major changes				
					cals.		*cals.*
73	−0.32	−4.62	−119	−17	102	−100	19
101	+0.08	+4.66	+108	+4	104	+91	17
65	−0.10	−3.86	−85	−5	80	−77	8
103	+0.13	+3.87	+113	+7	106	+78	35
66	+0.40	+5.24	+99	+22	77	+113	14
65	+0.77	+4.00	+144	+42	102	+103	41
Totals			668		571		134
			Minor changes in surface temperature				
43	−0.04	−0.72	−2	−2	0	−11	9
64	−0.03	−0.61	+1	−2	3	−13	14
57	−0.01	+0.56	+15	−1	16	+10	5
50	+0.12	+1.45	+17	+7	10	+32	15
85	−0.04	+1.15	+65	−2	67	+21	44
63	+0.02	+1.17	+61	+1	60	+24	17
198	−0.39	0	+12	−21	33	−14	26
143	−0.36	0	−25	−19	6	−13	12
Totals			198		195		142

while the formula gives much better agreement. No adjustment in the value for heat loss from the head could remove the discrepancies. When the change in surface temperature is less than 1.5°, the discrepancies by both methods are less serious, and there is less gain from the use of the formula.

Metabolism and average body temperature. Figure 7 shows the relations between the heat production, as measured by oxygen consumption, and the rectal temperature, and with the average body temperature calculated

by the formula respectively. There is somewhat better correlation with the latter. The scatter may be to some extent dependent on an incomplete attainment of the steady state, so that the formula was not strictly applicable. Consequently, when the bath temperature had been raised less than 30 minutes earlier, the points are indicated by triangles with the vertices uppermost, and vice versa. The distribution of these points suggests that in completely steady states, the scatter would be less. In addition to "chemical regulation" at low temperatures, a rise occurs with increasing average temperature (cf. data of Houghten et al., 1929), though it would be futile to calculate Q_{10} values. The minimum metabolism was

TABLE 4

Changes in rectal temperature following changes in bath temperature

BATH TEMPERATURES	RISE	INITIAL RISE IN RECTAL TEMPERATURE	CHANGE IN SAME DIRECTION—DELAY
			minutes
30.28 to 35.52	+5.24	0	10
32.26 to 36.26	+4.00	0	30
32.12 to 36.01	+3.89	−	16
33.83 to 35.00	+2.17	−	35
33.35 to 35.07	+2.28	−	48
35.07 to 37.01	+1.94	+	0
32.58 to 33.83	+1.25	−	25
33.58 to 35.10	+1.52	−	60
33.57 to 35.02	+1.45	0	28
33.00 to 33.57	+0.57	−	25
35.88 to 31.26	−4.62	+	35
36.00 to 32.12	−3.88	+	15
35.88 to 33.69	−2.19	+	55
35.87 to 34.06	−1.71	0	8
34.34 to 33.62	−0.72	+	45
35.92 to 35.11	−0.81	0	No fall
33.62 to 33.01	−0.61	+	50
35.11 to 34.76	−0.35	+	No fall

Delay measured from the start of change of bath temperature.

found at those bath temperatures which gave the minimum average temperature compatible with an absence of sensations of cold.

Paradoxical changes in rectal temperature. Paradoxical changes in rectal temperature, such as those seen in the exemplary protocols were the rule rather than the exception, so that the rectal temperature might not indicate correctly even the direction of change in average body temperature. When an actual fall of rectal temperature was not seen during warming, the rise was much delayed. A rise of rectal temperature on lowering the bath temperature was also common. The changes are listed in table 4, which also indicates (column 4) the delay between the start of the change in bath temperature and that of a rectal temperature change in

the same direction. This paradoxical change was described by Liebermeister and by Lefèvre (loc. cit.) who considered the possibilities of modified heat distribution but emphasized the metabolic factor on exposure to cold. Since the reflex change in conductivity is likely to take place rapidly, and owing to the thermal capacity of the tissues the new gradient will be established slowly, the product of the two may be expected to change initially in the opposite direction to the change in bath temperature. The paradoxical changes in both directions are then explicable in terms of changed conductivity through vasomotor adjustments, without necessarily any change in heat production. This may be demonstrated mathematically by consideration of a hypothetical physical model.

Physical model—mathematical considerations. In order to study the thermal kinetics of these changes, calculations have been made for a physical model used by one of us (Burton, 1934a) in explaining the thermal gradients of the body in the steady state. This model consists of a cylinder of material of uniform thermal conductivity in which heat is uniformly generated. Solution of the general differential equation for the flow of heat shows that in the steady state the distribution of temperature in the cylinder will be parabolic, being described by the equation

$$\theta_r = \theta_i - \frac{hr^2}{4K}$$

where θ_r is the temperature at radius r from the axis
θ_i is the temperature at the axis
h is the heat generated per unit volume, and
K is the thermal conductivity.

Such a distribution of temperature approximates that found by Bazett and McGlone (1927).

We must substitute the values for the rectal temperature (θ_i) and the surface temperature (θ_s) in one of the bath experiments to find the value of K, the effective thermal conductivity to make the model fit the experiment, i.e., $\theta_i = 36.5$°C., $\theta_s = 32.5$°C. Since a 70 kilo man may produce 70 kilo. Cals./Hr., h = 1/3600 Cal./cc./sec. Substituting, and assuming $r_s = 10$ cm., we find K = 0.0017 Cal./sq. cm./sec./°C./cm. for the effective conductivity.

The bath temperature in the experiment was then raised to 36.°C. so that the surface was forced to remain at that level. After about two hours the rectal temperature remained approximately constant at 37.°C. Assuming that this is the new steady state and substituting once more, we find that the cylinder must now have a conductivity K^1 equal to 0.0069 cal./sq. cm./sec./°C./cm., i.e., four times the original conductivity.

In order that we may find how this new steady state was reached and follow the temperatures within the cylinder in the transition period, a solution must be found of the general differential equation:

$$\frac{d\theta}{dt} = a^2\left[\frac{1}{r}\frac{d}{dr}\left(r\frac{d\theta}{dr}\right)\right] + b$$

where $a^2 = \frac{K^1}{s}$, $b = \frac{h}{s}$ and s is the specific heat such, that it fits the initial conditions.

We have to assume that the conductivity K^1 is constant throughout the transition (otherwise mathematical solution is beyond the capacity of the authors), that is,

that the reflex change of conductivity is completed at the start in a very short time. The solution is in terms of Bessel functions (J):

$$\theta = \theta_i - D - \frac{hr^2}{4K^1} + \Sigma\, e^{-\alpha_k^2} C_\kappa J_0\left(\frac{\alpha_\kappa r}{a}\right)$$

If the initial and final axial temps. are θ_i and θ'_i, those of the surface θ_s and θ'_s, then $D = \theta_i - \theta'_i$, and α_κ is given by the roots of the equation $J_0\left(\frac{\alpha_\kappa r_s}{a}\right) = 0$. The coefficients C_κ are found by the rule

$$C_\kappa = \frac{2}{\frac{\alpha_\kappa r_s}{a} J_1\left(\frac{\alpha_\kappa r_s}{a}\right)} \left\{ D + E\left(1 - \frac{4}{\left(\frac{\alpha_\kappa r_s}{a}\right)^2}\right)\right\}$$

where $E = (\theta'_i - \theta'_s) - (\theta_i - \theta_s)$.

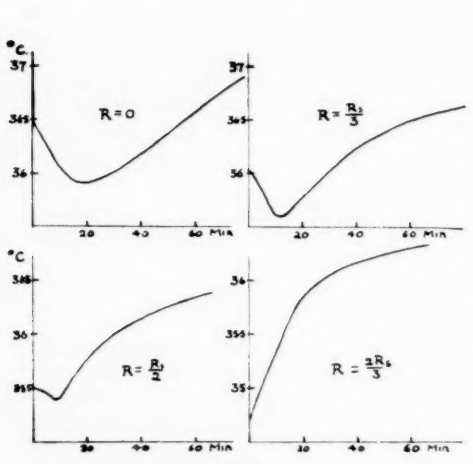

Fig. 8

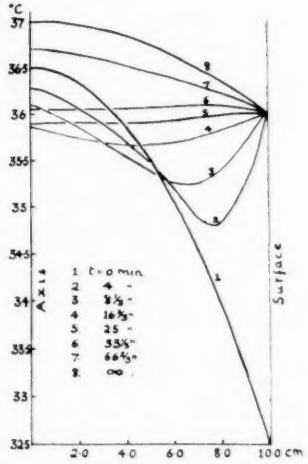

Fig. 9

Fig. 8. Time relations of thermal changes in a schematic cylinder.
Fig. 9. Thermal gradients in schematic cylinder at varying times.

Figure 8 shows how the temperature at different points in the cylinder changes with time, while figure 9 shows the temperature gradients in the cylinder at different times. The axial temperature initially falls and does not begin to rise for 20 minutes. Points nearer the surface have an initial fall of less magnitude, while those close to the surface do not show the initial paradoxical change. Whether or not the cylinder will accept heat from the bath depends on the gradient of temperature at the surface. Figure 9 shows that in this example the gradient is reversed for 25 minutes. In the actual experiment, on which the figures are based, the period of acceptance of heat by the subject was about 40 minutes. The correspondence is satisfactory considering that the choice of the radius of the cylinder, taken as 10 cm., affects the result greatly. It is difficult to estimate what value would best represent the average diameter of the "cylinders" of the immersed body. If r_s were taken as 7 cm. instead of 10 cm.,

the conductivities K and K^1 would be halved and the time to attain normal gradients become 50 minutes.

It is to be noted that a change in the thermal conductivity changes the time scale of the curves of figures 8 and 9 in the inverse ratio. The time taken to reach a steady state is longer, therefore, in a cold bath than in a hot bath.

Direct measurements of gradients in the skin up to depths of about 1 cm. had already been made under similar bath conditions (Bazett et al.), and demonstrated the presence of negative gradients. These are being reported separately.

SUMMARY

1. An insulated bathtub stirred and heated electrically, may be used as a calorimeter to measure the heat loss from the immersed surface. The surface temperature is then both constant and exactly known and the conditions of heat exchange may be measured. Such a calorimetric system has little lag, and measurements of exchange in short intervals are possible.

2. Physical regulation is able to maintain a balance between heat loss and normal heat production, when the rectal to surface temperature difference does not exceed 4°C. It is achieved by vasomotor alterations in the effective thermal conductivity which may change by a factor of about 6. In these experiments these changes were mainly of reflex origin.

3. Temperature differences exceeding 4°C. lead to an increased heat loss which exceeds normal heat production. Increased heat production (chemical regulation) is necessary but such increased heat production is accompanied by an increase in the effective thermal conductivity.

4. When the surface temperature is altered more than 1°C., the resulting changes in rectal temperature are inadequate to indicate even the direction of change in the average temperature of the body. An approximate measure is, however, given by a formula including both surface and rectal temperatures.

5. The heat production shows a minimum when the average body temperature is at the lowest level compatible with an absence of sensations of cold. The heat production shows a better correlation with average body temperature than with rectal temperature.

6. Paradoxical changes in deep body temperature are shown to be the necessary result of the changes in the conductivity index that accompany reactions to changes in temperature. The mathematical equations controlling such changes are given.

7. Some evidence is advanced of differences in the circulatory condition and consequent conductivity index in the same subject at different seasons of the year.

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THE PRODUCTION OF SYMPATHIN IN RESPONSE TO PHYSIOLOGICAL STIMULI IN THE UNANESTHETIZED ANIMAL

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Newton, Zwemer and Cannon (1931) observed a belated acceleration of the heart rate appearing three to four minutes after the onset of emotional excitement in unanesthetized cats with the adrenals and heart denervated. This acceleration was no longer present after the excision of the entire sympathetic system. The demonstration by Cannon and Bacq (1931) of the production of the hormone sympathin on stimulation of sympathetic nerves accounted for these observations.

Whitelaw and Snyder (1934) showed that sympathin is also liberated in the decorticate animal without adrenals during the periods of "sham rage." This condition is similar to emotional excitement in normal animals (Cannon and Britton, 1925).

In the present study an attempt has been made to show the production of sympathin in unanesthetized animals, using several methods for eliciting sympathetic activity: cold (Cannon, Querido, Britton and Bright, 1927) and hypoglycemia (Cannon, McIver and Bliss, 1924), in addition to emotional excitement. The nictitating membrane, sensitized by denervation, was used as an indicator to detect the presence of sympathin in the blood stream (Rosenblueth and Cannon, 1932).

METHOD. Cats were used. They were prepared by the aseptic removal of the right superior cervical sympathetic ganglion and section of the cervical sympathetic chain on the left side. The right adrenal was removed and the left denervated. A period of a week to ten days was allowed for the sensitization of the right nictitating membrane before any observations were made. The left membrane, only slightly sensitized to sympathin by the preganglionic denervation (Hampel, 1934), was used as a control.

To produce emotional excitement the cats were exposed to a barking, snapping dog at the door of the cage. This proved an adequate stimulus in the case of aggressive cats. In other instances the animals were tied on a board. If this alone was ineffective, release of all but one leg resulted in struggle. Insulin in the dosage of 3 units per kilogram, administered subcutaneously, was used to produce hypoglycemia. The animals went

into convulsions regularly in about 1.5 hours. The effect of cold was tested by keeping the animal for one hour in a room at 2°C.

RESULTS. Except for the periods of stimulation both membranes protruded equally over the eyes. Sympathin would be expected to induce contraction mainly on the right, highly sensitized side. These contractions were only considered positive when the visible portion of the right membrane was less than half that of the left control. No observations were made longer than one month postoperative, because of the possibility of regrowth of sympathetic fibers to the adrenals (Bacq and Dworkin, 1930).

Excitement. Exposure to a barking dog typically caused a retraction of the sensitized nictitating membrane, varying from half of the control to a mere rim. This reaction occurred in about 30 to 40 seconds and was accompanied by other signs of sympathetic activity such as erection of the hair and dilatation of the pupil. In spite of the continued presence of the dog, the membrane came back out after 2 to 3 minutes and usually protruded further than the control after the removal of the dog. At no time was there any retraction of the control membrane. One female cat which did not seem to be disturbed by the dog had no retraction of either membrane.

The results obtained by tying the cats down on an animal board were much the same as the above and had a similar time course. The animals did not usually struggle continuously for more than 2 or 3 minutes.

Hypoglycemia. Insulin in the dosage of 3 units per kilogram produced a profound lethargy within an hour, followed by nausea, vomiting, defecation, salivation, panting and dilation of the pupils. This was followed fairly promptly by generalized convulsions. During the period of other sympathetic manifestations and prior to the convulsions, the sensitized membrane retracted to a mere rim, but came back out after 2 or 3 minutes in spite of the fact that the animal went on into convulsions. It usually came further out than the control after intravenous glucose. In two rather sensitive preparations there were several periods of vomiting, each accompanied by a retraction of the membrane. In one cat that managed to get some food after the injection of the insulin, there were none of the typical manifestations of hypoglycemia, and neither membrane retracted.

Cold. Exposure to a temperature of 2°C. caused retractions of the sensitized membrane, usually 4 to 8 times during one hour. The membrane during retraction varied from about half the control to a rim and never remained in for more than 2 minutes. Retraction frequently followed a sneeze, yawn, or shivering. In one animal observed more than one month postoperative, sympathetic fibers had regrown to the control membrane. In this animal the re-innervated membrane retracted about

10 seconds before the sensitized one. The control membrane never retracted in experiments done within one month of operation.

Controls. As controls for the previous results 5 cats were prepared by sympathectomy (thoracic and abdominal) and the removal of the right superior cervical ganglion. The observations were made less than one month after the first stage of the sympathectomy because of the possibility of regrowth of the sympathetic fibers to the adrenals. Exposure to a barking dog and to a temperature of 2°C. caused no retraction of the sensitized membrane in 3 of these cats.

In the other 2 animals there was a slight retraction of the sensitized membrane to cold. Subsequent acute experiments under urethane anesthesia showed a rise in heart rate of from 8 to 12 beats per minute upon electrical stimulation of an afferent nerve. It was concluded that in these 2 cats the sympathectomy was not complete.

Discussion. Control experiments to show that the acceleration of the denervated heart after removal of the adrenals was not due to a rise of arterial pressure, increase of temperature, escape of adrenine from the denervated adrenal medulla or cortex, nor due to substances produced by the pancreas, gastro-intestinal mucosa, semilunar ganglia, pituitary body, male gonads, thyroids, parathyroids or active skeletal muscles were done by Newton, Zwemer and Cannon (1931). In the present observations the decentralized membrane was never seen to retract while the sensitized one regularly did so. Since the only physiological agents known to affect smooth muscle, whose effects are increased by denervation, are adrenine (excluded here) and sympathin, and since the retractions of the denervated membrane occurred under conditions favorable for the liberation of sympathin, it is concluded that the results observed were due to circulating sympathin. This is further shown by the absence of retraction of the denervated membrane after excluding circulating sympathin by total sympathectomy.

SUMMARY

After denervation of the adrenal glands various stimuli producing sympathetic activity, such as emotional excitement, hypoglycemia and cold, caused contractions of the sensitized nictitating membrane in unanesthetized cats.

Complete sympathectomy abolished these responses.

It is concluded that these contractions are due to circulating sympathin liberated in physiological conditions during sympathetic activity.

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DIETARY AND HEMATOLOGIC STUDIES AFTER GASTRECTOMY IN THE RAT

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Work previously reported from this laboratory showed that in the white rat gastrectomy caused anemia, cessation of growth, and, eventually, a decline in weight ending in death. The uniformity of these observations led Jung (1) to conclude that the gastrectomized rat differs from the gastrectomized man, dog, and cat in becoming anemic invariably, and that its nutrition is not normal.

It was evident that further attempts should be made to evaluate various procedures that might increase the survival time of the stomachless rat. In addition certain other observations on the bone marrow, spinal cord, etc., made on this and the previous series of gastrectomized rats will be reported.

METHODS. No operated rats were used unless they had survived more than 30 days and were able to eat the moderately rough stock diets without esophageal obstruction. By gastrectomizing 225 rats we obtained 45 which were satisfactory for our purpose and which were used in the dietary experiments to be reported here.

The method given by Jung and Jones (2) for gastrectomizing rats was improved. Twenty-four hours before operation the site of the midline abdominal incision was depilated with either potassium or barium sulphide, the alkali being removed immediately after by means of boric acid. Food was then withheld until after the operation. Under ether anesthesia and aseptic precautions, laparotomy was done. The stomach was removed between light wire clamps in one of which we were careful to include the large gastro-esophageal artery. For the anastomosis, either magnesium or aluminum cannulae were used; we did not find any advantage in one metal over the other. The original cannulae were of a kind intended by Payr for the anastomosis of blood vessels. We found that a shorter form was much preferable for our purpose; the best results were obtained with cannulae 4 mm. long, flanged, and 3 mm. in internal diameter.[1] The cannula was first tied into the esophagus with 00 catgut, the ligature being

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[1] Purchased from V. Mueller & Co., 408 S. Honore St., Chicago.

so placed as to include the large gastro-esophageal artery. Then the duodenum was drawn over the other end of the cannula and similarly tied. The abdomen was closed in two layers with catgut.

Water was placed in the cage immediately after the operation, and the rats drank freely and with evident benefit promptly after recovering from the ether. The diet for the first two weeks consisted of bread thoroughly soaked with either milk or tomato juice. During this period the rats generally lost weight. Autopsies on rats dying at various times showed that the cannulae underwent a gradual corrosion; they were rarely found after the 20th day. At about this time, also, the decline in weight generally stopped, and in some cases a rapid gain followed. The diet was then supplemented until by the 30th day the animals were on a stock diet.

Causes of death during the 30-day period were, in order of frequency, as follows: esophageal obstruction (70 per cent of deaths), local abscesses, hemorrhage, and cachexia. The deaths from obstruction would have been greatly reduced if we had discovered sooner that it was most commonly brought about by hair swallowed and caught at the anastomosis. This cause of obstruction was later completely eliminated by keeping the rats in separate cages.

Post-operative course. The post-operative growth-curves of the present series differed somewhat from those previously reported (1). Only a few rats of the present series ever regained their pre-operative weights. Generally there was a steady post-operative decline for about 2 weeks. After this, as normal eating habits were gradually resumed, the weight-curve levelled off or rose slightly to reach a plateau. The ensuing period of constant weight was often striking. Thus, rat 90 (YRF 2 male) from its 68th to its 123rd post-operative day never weighed less than 136 nor more than 149 grams; many other instances could be given. Frequently the weight of such a rat would be constant within one or two grams for a period of several days, so that the resulting curve was strikingly different from the "zig-zag" weight curve of a normal rat.

During the stage of constant weight, different animals presented different pictures. A few kept themselves clean, were active and inquisitive, and could be differentiated from normal rats only by their weight-curves and by their pale ears and eyes. No abnormalities of the teeth were ever seen. A few rats showed a curious coarsening of the hair; an example was rat 135 (PRF 1 male) whose hair (including the vibrissae) was noted after the 130th day as being long and dense. Many females (50 per cent) had persistent urinary incontinence; thus rat 99 (YRH 4 female) had periods of incontinence from the 84th to the 161st day, and again from the 201st to the 229th day, and again on the 235th day. In the intervals this rat was clean and well-nourished. Urinary incontinence was also seen occasionally (25 per cent), in the males. Priapism was frequent and per-

sistent (70 per cent) in the males; thus rat 135 (PRF 1 male) had extreme and continual priapism from the 23rd to the 50th day and again on the 148th. At these times the rat was nevertheless clean and lively.

Cause of incontinence and priapism. Genito-urinary symptoms were watched for with much interest since there was the possibility that they might be due to a degeneration of the spinal cord such as is seen in pernicious anemia. Such symptoms were frequent, as has been noted above. Attempts to obtain offspring by placing gastrectomized females with normal males, and gastrectomized males with normal females, never succeeded. Several females gastrectomized while pregnant gave birth to normal litters but refused to nurse their young. Finally four rats were sacrificed for the purpose of determining whether these abnormalities could be explained in terms of changes in the brain and cord. Neither rat 119, male, killed on the 87th day, nor rat 121, male, killed on the 101st day, had ever shown either priapism or incontinence. Rat 76 had priapism on the 13th day and had been incontinent for a few days before it was killed on its 103rd day. Rat 66 had had a period of priapism about its 74th day and had been incontinent for some time before it was killed on the 136th day. The brains and cords were instantly fixed. For the following information we are indebted to Dr. Arthur Weil (Department of Neurology) who stained the sections both for myelin and for cellular elements and examined the brains and the entire lengths of the cords. No signs of edema, inflammation, or degeneration were found; there were no histopathologic changes to indicate a subacute combined degeneration of the cord. The incontinence, priapism, and sterility of the stomachless rat cannot therefore be explained in the basis of anatomical changes in the brain or spinal cord.

Dietary procedures. Each procedure listed below was tried for periods of ten to twenty days and for several periods on not less than 2 nor more than 17 animals.

The procedures tried were:

1. Feeding stock diets I, II, and III (see table 1).
2. Feeding a bland pabulum developed in this laboratory for jejunal feeding in dogs (3).
3. Boiling the "rough" constituents of the diet.
4. Adding vitamin B (brewer's yeast) to the diet ($\frac{1}{2}$ gm. per day).
5. Adding vitamins A and D (cod liver oil) to the diet (1 gtt. per day).
6. Adding liver extract no. 343 (Lilly) to the diet (0.2 cc. per day).
7. Adding pancreatin (Merck) to the diet ($\frac{1}{4}$ gm. per day).
8. Adding banana powder to the diet (1 gm. per day).
9. Adding fresh vegetables to the diet.
10. Replacing the drinking water with iron water containing 3 grams of ferric ammonium citrate and 0.39 gram of copper sulphate per liter of tap water.

11. Replacing the drinking water with 0.4 per cent hydrochloric acid.

12. Subcutaneous injections of ferric ammonium citrate (5 cc., containing 1 mgm. of ferric ammonium citrate per cubic centimeter in 0.9 per cent sodium chloride solution) alone and together with liver extract no. 343 (0.2 cc. per day).

13. Subcutaneous injections of Phyone (1 cc. per day).[2]

14. Varying the daily diet.

This last procedure was apparently the best; but it did not produce satisfactory (i.e., continuous) gains in weight nor prevent decline in weight. It was noticed that nearly every marked change in diet produced a transient increase in weight.

Hematologic procedures. Blood examinations consisting of red blood cell count, hemoglobin by the Newcomer method, and determination of

TABLE 1

CONSTITUENTS	PER CENT COMPOSITION OF STOCK DIETS		
	1	2	3
Cracked yellow corn	60		
Ground corn		34	
Ground wheat		33	
Whole wheat flour			66
Powdered whole milk*	20	21	33
Powdered casein	16		
Linseed oil meal		7	
Alfalfa meal	3	2	
Vacuum dried liver meal		2	
Sodium chloride	0.5	0.5	1
Calcium carbonate	0.5	0.5	
	100.0	100.0	100.0

* Grateful acknowledgment is made to Nestlé's Milk Products, Inc. for supplying us gratis with "Lactogen" for this work.

mean cell diameter by direct measurement on fixed blood smears stained with Wright's stain were made every twenty days with few exceptions. Although all of the gastrectomized rats of this series developed a microcytic, hypochromic anemia which would respond to iron given orally and subcutaneously, its onset and severity were very variable in different animals. Poikilocytosis and anisocytosis increased with the severity of the anemia. It was shown by keeping 5 normal rats on a subnormal diet that the anemia of the gastrectomized rat was not due to low grade obstruction (see tables 2 and 3).

It was found that addition of vitamins A, B, and D to the diet in no way

[2] Generously supplied by Wilson Laboratories.

TABLE 2

SERIES	NUMBER OF DETERMINATIONS	RANGE	MEAN
Erythrocyte counts (in millions per cubic millimeter)			
Normal rats*	79	5.31– 9.76	7.87
Gastrectomized rats†	18	2.97– 9.77	5.72
Fasting rats‡	30	5.85–13.00	8.57
Hemoglobin (in grams per 100 cc.)			
Normal rats*	76	9.05–19.35	14.01
Gastrectomized rats†	18	4.30–14.93	8.96
Fasting rats‡	30	9.75–18.99	14.69
Mean diameters of erythrocytes (in micra)			
Normal rats*	7	6.00– 6.42	6.29
Gastrectomized rats†	7	4.45– 6.47	5.83
Fasting rats‡	0		

* Determinations made on 20 white rats of 5 different stocks.

† The counts included here were made between the 30th and 60th postoperative days on rats that lived longer than 80 days. All figures obtained at other times or after iron therapy on these rats or on rats that did not live more than 80 days are excluded.

‡ These determinations were made not less than 65 days after the normal rats had been placed on the subnormal diet.

TABLE 3

Brief, composite protocol of 5 rats kept on a subnormal diet of stock 1 to simulate weight curves of gastrectomized rats

DAYS	WEIGHT	RED BLOOD CELLS	HEMOGLOBIN
	grams		*grams*
0	163		
3	146	7.95	18.17
26	133	9.19	16.61
66	115	7.92	13.32
97	115	7.67	13.77
123	121	8.19	14.90
153	122	9.60	14.49
185	120	8.89	15.48
187	118	Fed ad libitum	
212	213		
227	226	9.14	16.16
247	235		

prevented the occurrence of the anemia. Addition of 0.4 per cent HCl to the diet increased rather than prevented the fall in hemoglobin. Re-

placing the drinking water of seven rats with iron water caused marked rises in hemoglobin in 5 rats, an average increase of 0.12 gram per 100 cc. per day for the 20 days on iron water, and no response, in fact, a continued fall in 2 rats. That the 5 responses were significant was shown by the fall in hemoglobin when the iron water was replaced by tap water. Ferric ammonium citrate subcutaneously caused in nearly every case an increase in red blood cell count, hemoglobin, and mean diameter. Curiously, the increase of mean diameter of the cell was proportionally more marked than the increase in Hb. In one rat (109 LFl male) the mean cell diameter increased from 4.45 to 7.55 micra. However, when the anemia was severe continuous subcutaneous iron therapy although causing some improvement could not restore the blood picture to normal. In one case orally administered liver extract (Lilly no. 343) had no effect. In two cases ferric ammonium citrate subcutaneously with orally administered liver extract (Lilly no. 343) in one case and subcutaneously administered liver extract (Lilly no. 343) in the other produced a greater remission than ferric ammonium citrate alone.

Bone marrow. The bone marrow of the femur was studied in 3 rats by Dr. F. D. Gunn, to whom we are indebted for detailed information which can only be summarized briefly here.

1. Rat 66 on its 69th day had far surpassed its preoperative weight and was in excellent condition with a red count of 8.62 million. However, its hemoglobin was only 6.64 grams, and the mean diameter of its erythrocytes was only 5.41 micra. It, therefore, had a severe microcytic and hypochromic anemia. On the 84th day daily injections of ferric ammonium citrate subcutaneously and daily administration of cod liver oil by mouth were started and continued beyond the 116th day. On this day the red count was 11.0 million, the hemoglobin 10.2 grams, and the mean diameter 5.98 micra. At this time esophageal stenosis began to develop, and the rat lost weight steadily. On the 136th day it still weighed more than at operation, but was killed for the sake of getting freshly fixed tissues. The chief differences noted between its marrow and that of normal rats was in the marked hyperplasia of the granulocytic elements, with increase of myelocytes and of immature polymorphonuclear cells and a relative paucity of erythrogenic elements. The interpretation was that the animal was suffering from a chronic infection and secondary anemia, although no evidence of infection was visible at autopsy.

2. Rat 76 on its 48th day was in excellent condition and had passed its preoperative weight. Its red count was 5.16 million, but the hemoglobin was only 4.5 grams and the mean diameter of the erythrocytes was 5.90 micra, below the lower limit of normal. It thus had a severe hypochromic and microcytic anemia. Iron and cod liver oil were given from the 52nd to the 70th day, the iron being increased gradually to the limits of tolerance. On the 83rd day the red count was 7.36 million and the mean diameter was 6.14 micra; but the hemoglobin was only 6.0 grams. Daily injections of iron were resumed, and daily additions of wheat germ to the diet were started on the 92nd day. On the 103rd day the rat was lively and its fur was clean in spite of a persistent urinary incontinence. It was killed for the sake of getting freshly fixed tissues, especially since it had both incontinence and priapism in its history. The neuropathologic findings (as stated above) proved to be negative.

In the bone marrow the erythropoietic foci were found to be extremely small and appeared to have been largely displaced by rapidly multiplying granulocytic elements. While no bacteria were found in the sections, the changes seen were typical of the hyperplasia usually found in infectious processes, although visible evidence of such a process was absent at autopsy.

3. Rat 22, which lived 320 days after operation, has too long and varied a history to be given in detail. It weighed 187 grams at operation, lost until on the 9th day it was down to 142 grams, and then started on a long course of steady gaining until on the 169th day it weighed 254 grams. It was used repeatedly in studies on the effect of iron injections. After the 282nd day it started on a steady decline which could not be stopped by iron, cod liver oil, or variations of diet. On the 320th day it weighed 159 grams; the red count was 8.75 million; hemoglobin was 9.10 grams; and the mean diameter of the erythrocytes was 3.83 micra—the most extreme microcytosis ever found by us. On this day it was killed. Examination of the bone marrow gave evidence of failure of maturation of both erythrocytic and granulocytic elements of about equal degree. The erythropoietic foci were small, though distributed fairly uniformly throughout the cellular part of the marrow. In or near each small group of undifferentiated cells there were a few normoblasts and the sinusoids contained an abundance of misshapen and various sized erythrocytes as well as a few normoblasts and many small nuclear fragments. Only occasionally were abnormal cells found in the form of megaloblasts or vacuolated erythroblasts. The megakaryocytes did not appear to be seriously altered. Altogether the changes strongly suggested "a primary anemia comparable to the pernicious anemia of the human" (Dr. Gunn).

Discussion. Although the failure of all the procedures to benefit materially the nutrition of the gastrectomized rat leaves us unenlightened as to the exact nature of the deficiency, these results do show that the arrested and finally failing growth of these gastrectomized rats was not due to traumatic enteritis, to loss of appetite due to monotonous stock diets, nor to deficiencies of vitamins, iron, growth hormone from the anterior lobe of the hypophysis, pancreatic enzymes, and hydrochloric acid. Low grade obstruction due to stenosis might have been a factor in causing growth failure; but this factor was ruled out as the cause of blood changes (see table 3). Bartonella infestation was ruled out as an etiological factor in the anemia by splenectomy. The organisms were never observed, although searched for, in the course of the numerous Price-Jones counts.

The hypoplasia found in the bone marrow, the fact that iron therapy only slightly benefited the severe anemias produced by gastrectomy and the fact that iron in conjunction with liver extract produced greater remission than iron alone even though liver extract alone had no effect on the anemia suggests that absence of the stomach in the rat interferes with either the preparation or the assimilation of substances which enter into the formation of the parent erythropoietic substances of Whipple (8) which later are utilized in hemoglobin formation by the bone marrow. From these findings further experiments with liver extract and iron should

be performed. However, the results will be rather difficult to interpret until it is learned how to control better the nutritional factor.

Apparently one important function of the rat's stomach is storage of ingested food for continuous discharge to the intestines as is shown by the weight curves of normal rats compared to the more constant weight curves of gastrectomized rats. These results lead us to believe that in the rat gastrectomy not only reduces the factor of safety in digestion, as was concluded by Ivy, et al. (4) after studying gastrectomized dogs, but so markedly impairs digestion that stunted growth, secondary anemia, and early death result.

The blood findings reported here agree with those previously reported for gastrectomized rats (5) (1). Oral and subcutaneous administration of iron was effective in causing partial remissions of the anemia produced by gastrectomy. This has also been found to be true in the gastrectomized pig (6) (7). Indeed, the gastrectomized pig shows growth and blood changes qualitatively similar to the growth and blood changes of the gastrectomized rat (6) (7), except in regard to the macrocytosis induced in some rats by the injection of iron.

CONCLUSIONS

1. The nutrition of rats was seriously impaired by gastrectomy.

2. This nutritional impairment was not definitely benefited by soft diets, high vitamin diets, varied diets, and addition of pancreatic enzymes, 0.4 per cent hydrochloric acid, liver extract no. 343 (Lilly), iron, banana powder, and fresh vegetables to the diet.

3. Gastrectomized rats developed a secondary anemia of the hypochromic, microcytic type.

4. This anemia, in most cases was improved by oral or subcutaneous administration of iron, and a relapse rapidly occurred if iron therapy was withheld.

Liver extract (Lilly no. 343) administered subcutaneously had no effect on the anemia in one case; but in two cases ferric ammonium citrate subcutaneously, with orally administered liver extract (Lilly no. 343) in one case and subcutaneously administered liver extract (Lilly no. 343) in the other, produced a greater remission than ferric ammonium citrate alone.

5. Absence of the stomach in the rat did not cause any changes in the spinal cord in any way suggestive of the changes seen in human pernicious anemia.

6. The bone marrow of the gastrectomized rat showed hypoplasia of erythropoietic foci with hyperplasia of granulocytic foci in two cases and hypoplasia of granulocytic foci in one case.

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AUGMENTATION OF THE GONAD STIMULATING ACTION OF PITUITARY EXTRACTS BY INORGANIC SUBSTANCES, PARTICULARLY COPPER SALTS[1]

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Several investigators have shown that a number of substances which have no demonstrable gonad stimulating ability when given alone, will increase the action of hypophyseal gonadotropic preparations when injected with them. Evans (1933) found this to be true of the blood and urine of several mammals and attributed the augmentation to the presence of a small amount of gonadotropic substance similar to that found in the urine of pregnant women. Maxwell (1934) showed that zinc sulfate increased the action of pituitary extracts and believed that this was due to a decrease in the rate of absorption of the active material. Cassida (1935) reported that the augmenting action of the blood of cattle was a property of the formed elements rather than of the plasma. Hellbaum (1936) demonstrated the presence of augmenting substances in human urine, horse thyroid, beef liver, milk and lemon juice, while Cole and Hart (1934) and Saunders and Cole (1935, 1936) found that pregnant and non-pregnant mare serum, casein and egg albumin all enhanced the ovarian response to pituitary extracts. They, like Maxwell, believed that the increased action was due to a decrease in the rate of absorption. Friedman (1934) reported that an extract of alfalfa meal produced ovulation when injected intravenously into mature rabbits during oestrus.

We have found that aqueous pyridine extracts of dried brewer's yeast also will increase the gonadotropic activity of hypophyseal preparations in normal immature female rats. The hypophyseal extracts used were the follicle stimulating preparation (F.S.H.) and the unfractionated extract (F.S.H. plus L.H.) prepared by methods previously reported (1934). These extracts were injected into 22-day old rats in doses which alone caused very little increase in ovarian weight. The rats were injected twice daily for three days and autopsied 24 hours after the last injection. The same dosage, combined with various amounts of yeast extract, produced much greater ovarian development than that elicited by the gonadotropic

[1] Aided by a grant from the National Research Council, Committee on Problems of Sex.

preparation alone. The quantitative increase in ovarian weight was unaccompanied by any qualitative change since the F.S.H. caused the development of follicles with or without yeast while the extract containing both F.S.H and L.H. produced luteinized ovaries in both instances (tables 1 and 2).[2]

The yeast extract, similar to Friedman's alfalfa extract, caused ovulation in mature rabbits. Doses of 10 to 15 grams equivalent were given intravenously at a single injection and the ovaries 48 hours later showed typical ovulation points and a number of hemorrhagic follicles. The yeast extracts were not toxic at the doses given.

The augmenting substance (or substances) in the yeast extract was very stable to heat. Boiling for several hours had no effect on its activity, nor did heating the dried extract at 100° to 110°C. impair its ability to augment gonadotropic preparations.

A sample of the yeast powder was ashed, an acid extract was made of

TABLE 1

Effect of yeast preparations on ovarian response to F.S.H.

AMOUNT OF YEAST PREPARATION (GRAM EQUIVALENT)	YEAST EXTRACT	YEAST ASH
	Ovarian weights (mgm.)	
None	16	16
1	37	35
0.5	30	33
0.25	26	24
0.1	20	21

TABLE 2

Effect of yeast preparations on ovarian response to F.S.H. plus L.H.

AMOUNT OF YEAST PREPARATION (GRAM EQUIVALENT)	YEAST EXTRACT	YEAST ASH
	Ovarian weights (mgm.)	
None	18	18
1.0	51	76
0.5	48	53
0.25	32	36
0.1	26	24

the ash and the neutralized solution tested as before. The acid-soluble ash seemed to be as active as the yeast extract in producing augmentation. It was evident therefore that at least a part of the augmenting ability was due to the inorganic constituents of the yeast.

Since qualitative tests showed that the yeast ash contained, among other things, a considerable amount of copper and iron, and since Maxwell had already reported the augmenting ability of zinc, we investigated the action of several inorganic salts. Of these, those of copper produced the greatest effect. The augmentation elicited by zinc salts was much less than that of copper while the effect of iron salts was less than that of zinc. Manganese, aluminum and calcium salts produced no or at most only slight augmentation.

The quantitative results for copper and zinc salts were very different in that copper produced greater augmentation. The qualitative results,

[2] The ovarian weights given in the tables in the paper are the averages for at least six animals in each case.

however, were quite similar as neither salt modified the characteristic effects of the pituitary extract with which it was combined. These observations led to a detailed comparative study of the action of salts of these two metals. The experimental procedure was the same as that used in testing the yeast extracts but in addition to normal rats, hypophysectomized rats were used. These were hypophysectomized when they were 28 days old and the experiments were started 48 hours after the operation.

Zinc sulfate, combined with F.S.H. or with F.S.H. plus L.H., increased the activity of both preparations when injected into normal 22 day old rats or into hypophysectomized rats (table 3). One milligram of zinc sulfate, injected into normal rats in combination with a constant amount of F.S.H. apparently produced a maximum augmentation, as the addition of more salt caused no further increase. Combined with F.S.H. plus L.H., the salt also produced augmented results, the increase being greater than

TABLE 3

Effect of zinc sulfate on ovarian response to F.S.H. and to F.S.H. plus L.H.

	$ZnSO_4$ (mgm.)				
	None	1	3	6	10
	Ovarian weights (mgm.)				
*F.S.H.	16	26	25	26	28
*F.S.H. plus L.H.	19	21	45	47	
†F.S.H.	13		22		
†F.S.H. plus L.H.	16		46		

* Normal immature rats.

† Rats hypophysectomized 48 hours before injections were started.

when combined with F.S.H. alone. The results of similar experiments on hypophysectomized rats were comparable to those for the normal.

Copper salts increased the action of F.S.H. and of F.S.H. plus L.H. more than did zinc sulfate, when tested in normal immature rats (tables 4 and 5). Combined with the same amount of F.S.H., 0.1 mgm. copper sulfate accentuated the ovarian response approximately the same as 1.0 mgm. zinc sulfate. By increasing the amount of copper salt, the same dosage of F.S.H. increased the ovarian weight to 55 mgm. while with the zinc salt the limit apparently was reached at 26 mgm. Likewise, when added to F.S.H. plus L.H., copper salts were more effective than those of zinc. Five milligrams of copper acetate increased the effectiveness of the extract approximately 2000 per cent, $(96–15) - (19–15) / (19–15) \times 100$, while the maximum augmentation obtained with zinc salt was much less. The effects of zinc and copper salts were similar in that they were more effective when injected with a combination of both pituitary hormones than with

F.S.H. alone. Their action was also similar to yeast extract in that neither of the two kinds of salts altered the qualitative ovarian response to the extracts.

In hypophysectomized rats the addition of copper acetate to F.S.H. plus L.H. elicited an accentuated response in ovarian weight comparable to that obtained in normal immature rats (table 6). However, in such animals, copper salts did not increase the effectiveness of F.S.H., the response being the same quantitatively and qualitatively with or without copper (table 7).

TABLE 4

Effect of copper salts on the ovarian response to F.S.H. (normal rats)

	AMOUNT (MGM.) SALT				
	None	0.1	0.5	1.0	5.0
	Ovarian weights (mgm.)				
$CuSO_4$	15	25	28	45	52
$Cu(C_2H_3O_2)_2$	15	26	50	42	55
$CuCl_2$	15	21	30	40	56

TABLE 5

Effect of copper salts on the ovarian response to F.S.H. plus L.H. (normal rats)

	AMOUNT (MGM.) SALT				
	None	0.1	0.5	1.0	5.0
	Ovarian weights (mgm.)				
$CuSO_4$	19	24	67	70	65
$Cu(C_2H_3O_2)_2$	19	26	46	62	96
$CuCl_2$	19	23	41	58	86

TABLE 6

Effect of copper acetate (0.5 mgm.) on the response of the ovaries of hypophysectomized rats to F.S.H. plus L.H.

F.S.H. PLUS L.H. (MGM.)	WEIGHT OF OVARIES (MGM.), NO Cu	WEIGHT OF OVARIES (MGM.), Cu
0.75	23	60
1.5	30	88
3.0	42	60
6.0	63	90

TABLE 7

Effect of copper acetate (0.5 mgm.) on the response of the ovaries of hypophysectomized rats to F.S.H.

F.S.H. (MGM.)	WEIGHT OF OVARIES (MGM.), NO Cu	WEIGHT OF OVARIES (MGM.), Cu
0.5	17.5	18
1	21	21
2	24	25
8	32	31
16	42	40

These results bring out an important difference in the probable mechanism of augmentation produced by zinc and copper salts. The fact that copper increased the ovarian response of normal rats to F.S.H. but did not do so in hypophysectomized rats would seem to eliminate the possibility of explaining the increased action by assuming that the rate of absorption is reduced with consequent greater efficiency in the utilization of the active substance. This does not seem to be the true explanation as one would expect the salt to slow down absorption in hypophysectomized as well as in normal animals. Zinc sulfate, however, does increase the action of

F.S.H. in both types of test animals. The effect of zinc salts on F.S.H. action is quite similar to that produced by tannic acid which clearly decreases the rate of absorption. Zinc salts, therefore, as first demonstrated by Maxwell, apparently produce their effects by prolonging absorption, but it does not seem possible to explain the action of copper salts on this basis.

It was found also that intravenous injections of copper salts into mature rabbits in heat would produce ovulation within 24 to 48 hours. These results differed from those obtained by injecting pregnancy urine or pituitary extracts in only one respect, namely, that the interval between the injection of copper salts and ovulation was longer than that usually required for gonadotropic extracts. Ovulation was not observed in any case within the first twelve hours following injection. Some ovulated in 24 hours while others required a longer period. Two rabbits which received 5 mgm. of copper acetate failed to ovulate but the follicles were hemorrhagic and several were cone-shaped and appeared ready to rupture. Three received 10 mgm. of the same salt and the ovaries of each contained from 5 to 8 ovulation points. Two received 15 mgm. with results similar to those receiving 10 mgm. Zinc sulfate, chloride, or acetate in doses up to 25 mgm., did not produce ovulation or hemorrhagic follicles in seven animals which again demonstrates a difference in the effects of copper and zinc.

Discussion. Augmentation resulting from a combination of gonadotropic substances and yeast extracts or inorganic salts does not seem to be of the same nature as that obtained when L.H. is combined with F.S.H. (Fevold and Hisaw, 1934). Luteinizing hormone combined with F.S.H. results in a type of augmentation which is apparently due to the two gonadotropic substances acting synergistically. This is evidenced by the fact that the augmentation is accompanied by a change in the character of the response as the ovaries contain only follicles when F.S.H. acts alone and chiefly corpora lutea when F.S.H. and L.H. act together. Yeast extract, zinc and copper salts on the other hand augment the action of F.S.H. and of F.S.H. plus L.H. without altering the qualitative action of either preparation.

Maxwell (1934) questions the duality of the pituitary gonadotropic hormones, because of the reported alteration in the ovarian response to his extracts when combined with $ZnSO_4$. He intimates that our evidence for two hormones may be due to the fact that our extracts were treated with tannic acid. However, a perusal of our work will show that tannic acid does not change the qualitative response and that a difference in absorption rate is not an adequate explanation of the results (Fevold *et al.*, 1933). Likewise, using our preparations which contain both F.S.H.

and L.H. in approximately their normal balance, no change in the character of the response was observed when $ZnSO_4$ was added.

It seems possible that copper salts may accomplish their effects by catalyzing the synergistic action between F.S.H. and L.H. By this assumption we can explain the fact that copper salts increase the action of F.S.H. in normal rats but do not do so in hypophysectomized rats. Subminimal amounts of gonadotropic substances are apparently present in the blood of normal rats[3] and the augmentation caused by copper when injected with F.S.H. may be due to the facilitation of the synergistic action of the L.H. already in the blood with the injected F.S.H. In hypophysectomized animals, the bodies of which are presumably free of the gonadotropic principles, no effect is produced by copper salt in combination with F.S.H. since no L.H. is present. If, however, L.H. is added to the F.S.H. copper salt combination the results for hypophysectomized rats are similar to those obtained for normal rats.

Ovulation in a normal, mature rabbit is generally believed to take place when the gonadotropic substances are increased to the threshold for ovulation. This is brought about apparently by nervous stimulation of the pituitary as a result of mating, or ovulation may be induced experimentally by injecting the pituitary gonad-stimulating factors. Copper salts probably cause ovulation by increasing the effective activity of the gonad-stimulating hormones present in the blood and thus the threshold for ovulation is attained. Zinc salts, which presumably cause augmentation in rats by decreasing the absorption rate, apparently have no effect on the hormones already in the blood, at least not to the extent of increasing their activity to the level required for ovulation.

SUMMARY

The gonadotropic action of follicle stimulating hormone (F.S.H.) and of F.S.H. plus luteinizing hormone (L.H.) on the ovaries of immature rats was increased when combined with yeast extract, yeast ash, zinc and copper salts. Zinc salts also augmented the action of F.S.H. and of F.S.H. plus L.H. when tested on hypophysectomized rats. Copper salts augmented the action of F.S.H. plus L.H. in hypophysectomized rats but had no effect on the activity of F.S.H. alone in such animals. Yeast extracts and copper salts caused ovulation in mature rabbits while the salts of zinc were ineffective in eliciting this response.

Zinc salts probably produce their effect by decreasing the rate of absorp-

[3] The presence of gonadotropic substances in immature rats is indicated by the fact that atrophy of the gonads takes place when the pituitary is removed and also by the partial luteinization of the ovaries of a small percentage of immature rats even though "pure" F.S.H. is administered.

tion of the active material. The activity of copper salts apparently cannot be explained on this basis but may be due to a catalytic action in the synergistic interaction of F.S.H. and L.H. in ovarian development.

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THE EFFECTIVENESS OF CARBON DIOXIDE IN COMBATING THE CHANGES IN VISUAL INTENSITY DISCRIMINATION PRODUCED BY OXYGEN DEFICIENCY

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In 1898 Mosso reported on experiments in the low pressure chamber which indicated that small concentrations of CO_2 (2 to 5 per cent) increased the resistance of his experimental subjects to lowering of the barometric pressure. Similar observations on animals were reported by Margaria (1928) and Talenti (1930) and recently Childs, Hamlin and Henderson (1935) found, on Pike's Peak, that mountain sickness could be alleviated by the inhalation of CO_2. The only reported experiments in which, instead of lowering the barometric pressure, the partial pressure of oxygen was reduced by diluting the air with nitrogen failed to give the typical effect of CO_2 (Margaria). Since Gellhorn and Janus (1936), in their studies on the influence of oxygen deficiency on body temperature, found, in agreement with the older literature, no difference whatever between the effects of lowering the barometric pressure and diluting the air with nitrogen if the partial pressure was the same, it seemed to be of considerable interest to investigate:

1. Whether CO_2 is effective in combating the effects of oxygen deficiency if the latter is produced by air-nitrogen dilution.

2. To investigate this problem in regard to the human central nervous system where the effects due to oxygen deficiency are greatest and can be studied quantitatively, as the recent investigations of Gellhorn (1936) and Gellhorn and Spiesman (1935) show.

In the present paper the influence of 3 per cent CO_2 on the effects of oxygen deficiency on visual intensity discrimination was investigated. Fifteen experiments were carried out with uniform results. The experimental subjects inhaled the gas mixtures from Douglas bags, which, in one group of the experiments, contained 8 to 9 per cent oxygen-nitrogen mixtures, whereas in a second group, the same oxygen-nitrogen mixtures plus 3 per cent CO_2 were inhaled. As to the technique, the paper by Gellhorn (1936) may be consulted.

RESULTS. A typical result is given in figure 1, showing the effects of pure oxygen deficiency and oxygen deficiency plus 3 per cent CO_2 on the

visual intensity discrimination of two subjects. In one case (heavy line) the effect of oxygen deficiency was completely offset by 3 per cent CO_2, although a very marked decrease was obtained in the corresponding experiment with oxygen deficiency, in which no CO_2 was inhaled. The other subject (dash line) showed a decrease in visual intensity discrimination in both instances, but the effect was considerably smaller in the presence of 3 per cent CO_2; and, furthermore, the original threshold was reached in the latter part of the experiment, although the subject continued to breathe $8\frac{1}{2}$ O_2 + 3 per cent CO_2.

Table 1 shows some typical experiments with five subjects in which the

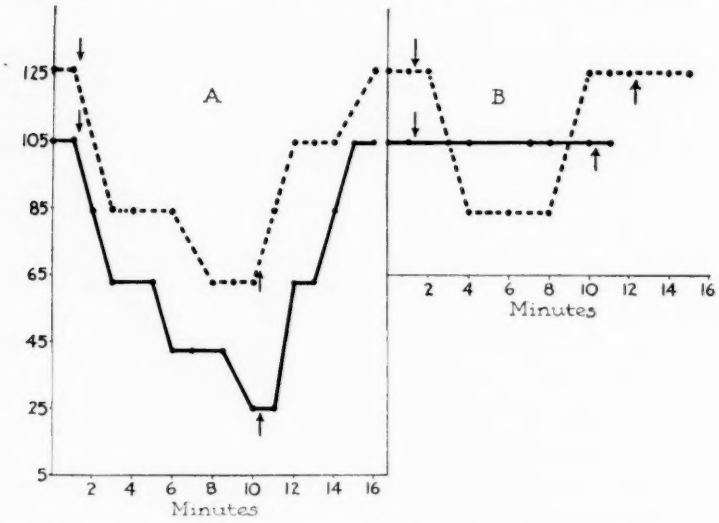

Fig. 1. Ordinate: Reciprocals of the threshold for visual intensity discrimination. Abscissa: Time in minutes. In the figure 1A and 1B $8\frac{1}{2}$ O_2 and $8\frac{1}{2}$ O_2 +3 per cent CO_2 respectively were inhaled between the two arrows by two different subjects.

oxygen concentration varied between 8 and 9 per cent. The figures show, as a characteristic result, that the effect of oxygen deficiency is either greatly diminished or completely absent in the presence of 3 per cent CO_2. In the former case a complete compensation is observed in the latter part of the experiment, that is ordinarily after 4 to 8 minutes. In contradistinction to this, the effect of oxygen deficiency alone on visual intensity discrimination shows a progressive character, as the figure and table, as well as the experiments of Gellhorn (1936) indicate.

A series of control experiments was carried out with 3 per cent CO_2 in air. No effect whatever was noted in these experiments. This is not

surprising in view of the fact that even with 6 per cent CO_2 the effects on visual intensity discrimination are rather slight (compare Gellhorn, 1936).

In agreement with these findings are the subjective symptoms. Practically no subjects had any complaints in the presence of CO_2, although the corresponding oxygen deficiency experiment without CO_2 led to feeling of warmth, dizziness, etc. It may, however, be emphasized that the results of oxygen deficiency are not simply due to the general interference with the

TABLE 1

The influence of CO_2 on the change in intensity discrimination produced by O_2-lack

SUBJECT	CONTROL	EXPERIMENTAL CONDITION				CONTROL		
He.	1.* 126†	9% O_2	2. 84	5. 84	8. 63	1. 84	3. 105	6. 126
	2. 126		3. 84	7. 63	9. 63	2. 105	4. 105	7. 126
He.	1. 126	9% O_2	1. 126	5. 84	9. 126	1. 126	3. 126	
	2. 126	+3% CO_2	3. 84	7. 84	10. 126	2. 126		
H.	1. 147	8% O_2	1. 147	3. 126	5. 105	1. 126	3. 147	
	2. 147		2. 147	4. 126	6. 105	2. 126	4. 147	
H.	1. 147	8% O_2	1. 147	3. 126	5. 147	1. 147		
	2. 147	+3% CO_2	2. 147	4. 147	6. 147	2. 147		
K.	1. 105	8% O_2	1. 105	4. 84	6. 63	1. 105	3. 126	6. 105
	2. 105		3. 84	5. 84	7. 84	2. 126	4. 105	
K.	1. 105	8% O_2	1. 105	4. 105	6. 105	1. 126	3. 105	
	2. 105	+3% CO_2	3. 105	5. 105	7. 105	2. 126	4. 105	
J.	1. 126	$8\frac{1}{2}$% O_2	1. 105	5. 105	8. 105	1. 126		
	2. 126		3. 84	6. 105		2. 126		
J.	1. 126	$8\frac{1}{2}$% O_2	1. 126	5. 126	7. 126	1. 126		
	2. 126	+3% CO_2	3. 126	6. 126	8. 126	2. 126		
R.	1. 105	$8\frac{1}{2}$% O_2	2. 105	4. 63	6. 63	1. 105		
	2. 105		3. 63	5. 63	8. 63	2. 105		
R.	1. 105	$8\frac{1}{2}$% O_2	2. 105	4. 105	7. 105	1. 105		
	2. 105	+3% CO_2	3. 105	6. 84	8. 105	2. 105		

* Time in minutes.

† The figures represent reciprocals of the visual intensity discrimination.

well being of the experimental subject, since the same characteristic differences have been observed between the pure oxygen deficiency experiments and those in which 3 per cent CO_2 in addition to the same O_2-concentration was inhaled, although practically no subjective symptoms were reported in either of the experiments.

The observations show clearly that the beneficial effect of CO_2 on oxygen deficiency occurs in oxygen-nitrogen mixtures at normal atmospheric

pressures. The most probable interpretation seems to be that the CO_2 effect is due to:

1. The improvement of the function of the brain due to a circulatory adjustment. In favor of this assumption are the observations by Lennox and Gibbs (1932) and others cited by Gellhorn and Spiesman (1935) that CO_2 causes a dilatation of cerebral vessels, thereby improving the circulation of the brain.

2. The shift to the right in the oxygen dissociation curve of the blood, thereby increasing the rate at which oxygen is given off to the tissues.

3. The improved muscular tonus resulting even from small concentrations of CO_2 (Henderson and collaborators, 1936) which increases the venous return to the heart.

The rôle of CO_2 in circulatory adaptation to oxygen deficiency is shown further by the fact that small amounts of CO_2, which by themselves are without influence on the blood pressure of anesthetized dogs, produce, in the presence of oxygen deficiency, a distinct increase in blood pressure, as our own unpublished observations indicate.

It may be mentioned that experiments which will be published elsewhere have shown that the effect of CO_2 in alleviating the effects of oxygen deficiency was also obtained in regard to certain more purely psychic functions.

SUMMARY

The effects of breathing 8 to 9 per cent oxygen on the visual intensity discrimination in man can be either completely removed or greatly diminished by small concentrations of CO_2 (3 per cent) which in themselves have no effect on the sensory function investigated. It is believed that this effect is due to the circulatory improvement induced by CO_2 under oxygen deficiency.

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THE RÔLE OF THE DUODENAL SECRETIONS IN THE PREVENTION OF EXPERIMENTAL JEJUNAL ULCER

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When the duodenal secretions are drained into the lower ileum and the jejunum anastomosed to the pyloric end of the stomach (Exalto, Mann-Williamson operation) chronic ulcers form in the jejunum just below the suture line in from one to four months in 95 to 100 per cent of animals (1). Mann (2) found that if the duodenum was drained into the jejunum, just below the suture line, ulcer formation was prevented. Matthews and Dragstedt (3) showed that drainage of the duodenum into the jejunum even as far as 40 cm. below the gastrojejunal anastomoses would prevent ulcer. Mann (2) and Morton (4) showed that existing experimental jejunal ulcers could often be healed by draining the duodenal secretions over the ulcer area. These observations agree in showing that jejunal ulcer can be prevented or healed if the duodenal secretions are drained into the jejunum below the gastrojejunostomy.

When the duodenal secretions are drained into the stomach the findings have not been so uniform. Mann (5) and McCann (6) found that ulcer formation was not prevented. Ivy and Fauley (7), however, found a greatly reduced incidence of ulcer (2 out of 12 animals). The findings of Mann and McCann are difficult to explain and raise the question as to why the duodenal secretions should protect the jejunum from ulcer when drained directly into the jejunum but not when drained into the stomach. Since no adequate explanation has been offered, the present experiments were planned to re-investigate the problem.

METHODS. In the work of Mann (5), McCann (6) and Ivy and Fauley (7) the duodenum was drained into the stomach by an end to side anastomosis. With this technique the stoma is often very small and it is possible that free drainage of the duodenal secretions into the stomach is interfered with. In addition the duodenum does not receive direct mechanical and chemical (acid chyme) stimulation. When mechanical and chemical stimulation is lacking we have observed that the duodenal secretions, especially the succus entericus, may be scanty in amount but can be

greatly increased by adequate stimulation. Because of these facts the method of draining the duodenum into the stomach was modified so that almost the entire duodenum was opened into the anterior wall of the stomach by a side to side anastomosis with a stoma 5 to 6 inches long. Under these conditions the duodenum virtually formed part of the anterior wall of the stomach proximal to the pyloric region, thus insuring adequate stimulation and free drainage. Often a small portion of the distal duodenum could not be used, this and the upper 6 or 8 inches of the jejunum were resected and the distal part of the jejunum anastomosed to the pylorus, end to end. Intestinal clamps and silk sutures were used because these are often cited as predisposing factors in ulcer formation.

A second series of experiments was performed in which the duodenum was drained into the jejunum a few inches below the gastrojejunostomy by a side to side anastomosis with a very long stoma.

The gastric analyses were performed with two per cent Liebig's extract test meal containing 15 mgm. of phenol red per liter (8).

RESULTS. A. *Ulcer formation.* In agreement with the work of Mann and Matthews and Dragstedt no ulcers were found in any of the four dogs in which the duodenum was drained into the jejunum below the gastrojejunal anastomosis. One dog is still living (90 weeks) and is in excellent condition (table 1).

Six dogs in which the duodenum was drained into the stomach lived from 3.3 to 39.3 weeks and of these, five were entirely negative for jejunal ulcer or jejunitis at autopsy (table 1). One dog died from acute portal thrombosis 38.6 weeks after operation. This dog was in shock for several hours before death and at autopsy a small, very superficial erosion was found in the jejunal mucosa just below the suture line. Microscopic examination showed this to involve only the superficial layer of the mucosa and to show no signs of chronicity. Considering the long duration of life after operation and the mode of death, this superficial erosion can probably be safely assumed to be a terminal event not directly related to the experimental conditions. Although the series is small, the results appear to justify the conclusion that the duodenal secretions when drained into the stomach will protect the jejunum from ulcer just as efficiently as when drained directly into the jejunum.

When the duodenum was drained into the jejunum the condition of the animals was in every way normal and the appetite remained good. When the duodenum was drained into the stomach the animals failed to remain in a normal condition, there was usually marked weight loss and general debility. Anorexia was often present, being continuous in some dogs and intermittent in others. Both of the animals which survived for over nine months showed marked weight loss; in dog VI the appetite was ravenous

throughout the entire period while in dog V a ravenous apetite was interrupted on several occasions by periods of complete anorexia.

TABLE 1

DOG NUMBER	PERIOD OF SURVIVAL	ULCER AT AUTOPSY	REMARKS	TYPE OPERATION
	weeks			
I	3.3	None	Died. Marked anorexia and weight loss	
II	4.4	None	Died. Marked weight loss. Moderate anorexia for all food but meat	
III	8.4	None	Died. Appetite fair. Moderate weight loss	Mann-Williamson operation with duodenal drained into the stomach
IV	8.9	None	Killed with ether because of severe red mange. Appetite good. Marked weight loss	
V	39.3	None	Strangled from food in larynx. Appetite ravenous with occasional short periods of anorexia. Moderate weight loss	
VI	38.6	Small, very recent, involving only superficial layers of mucosa	Died. Portal thrombosis. Appetite ravenous. Marked weight loss	
VII	4.0	None	Killed with ether because of severe Jacksonian epileptic seizures of unknown etiology since operation. Appetite good	
VIII	14.4	None	Killed with ether. Hair balls in duodenal pouch and jejunum causing obstruction. Condition good until this time. Appetite good	Mann-Williamson operation with duodenum drained into jejunum
IX	40.4	None	Died suddenly. No cause found. Condition excellent. Appetite good	
X	90.0	No evidence of ulcer	Still living. Appetite good. Condition excellent	

B. *Gastric analysis.* In table 2 gastric analyses are shown on one dog with each type of operation.

1. *Dog V. Mann-Williamson operation with the duodenum drained into the stomach.* The resting stomach was always found to contain enormous

amounts of heavily bile stained fluid, as much as 1000 cc. were removed at times. When the acidity curve was studied with a two per cent Liebig's extract test meal, the acidity of the gastric contents due to acid secretion

TABLE 2

MEAL. MGM. CHLORIDE PER 100 cc.	GASTRIC SAMPLE, MGM. PER 100 cc.			GASTRIC CONTENTS, MGM. PER 100 cc.			GASTRIC SECRETION, MGM. PER 100 cc.			TOTAL FLUID	ACID FLUID	NON-ACID FLUID	NEUTRAL CHLORIDE / NON-ACID FLUID	BILE	VOLUME OF SAMPLE, CC.	TIME, HOURS	
	Total chloride	Neutral chloride	P.S.P., per cent	Extra total chloride	Extra neutral chloride	Extra acid chloride	Total chloride	Neutral chloride	Acid chloride								
Total..... 215	261	189	79	91	52	39	433	248	185	21	7	14	3.7	++++	35	½	
Neutral.. 173	289	195	72	134	70	64	479	250	229	28	11	17	4.1	++++	35	1	
Acid..... 42	332	190	62	199	83	116	523	218	305	38	19	19	4.4	++++	36	1½	
	316	212	52	204	122	82	425	254	171	48	14	34	3.6	++++	35	2	
	346	226	54	230	132	98	500	287	213	46	16	30	4.4	++++	443	2½	Dog V. Mann-Williamson operation with duodenum drained into stomach
Total..... 232	275	191	76	99	52	47	413	217	196	24	8	16	3.3	+++	35	½	
Neutral.. 183	309	201	63	163	86	77	440	232	208	37	13	24	3.6	+++	35	1	
Acid..... 49	308	210	40	215	137	78	358	228	130	60	13	47	2.9	+++	36	1½	
	322	226	64	174	109	65	484	303	181	36	11	25	4.4	+++	573	2	
Total..... 250	288	193	83	81	49	32	476	288	188	17	5	12	4.1	+	37	½	
Neutral.. 173	305	206	76	115	75	40	479	312	167	24	7	17	4.4	+	36	1	
Acid..... 77	329	215	61	177	110	67	453	282	171	39	11	28	3.9	++	35	1½	
	342	260	54	207	166	41	450	361	89	46	7	39	4.3	+++	950	2	
Total..... 218	263	174	86	75	14	61	535	100	435	14	10	4	3.5	++	35	½	
Neutral.. 186	290	182	78	120	37	83	545	168	377	22	14	8	4.6	+++	35	1	
Acid..... 32	334	162	69	184	34	150	594	111	483	31	25	6	5.7	+++	35	1½	
	366	175	61	233	62	171	596	159	437	39	29	10	6.2	+++	180	2	
Total..... 222	265	162	94	56	0	56	934	0	934	6	6	0		0	35	½	
Neutral.. 176	285	173	89	87	16	71	790	146	644	11	11	0		0	35	1	Dog X. Mann-Williamson operation with duodenum drained into jejunum
Acid 46	312	164	86	121	13	108	864	93	771	14	14	0		0	35	1½	
	358	184	82	176	40	136	980	222	758	18	18	0		0	852	2	
Total..... 224	307	155	74	141	28	113	543	108	435	26	19	7	4.0	trace	35	½	
Neutral.. 172	344	174	61	207	69	138	531	177	354	39	23	16	4.3	trace	35	1	
Acid..... 52	384	138	62	245	31	214	645	82	563	38	36	2	15.5	trace	34	1½	
Total..... 204	316	152	66	181	40	141	533	118	415	34	24	10	4.0	0	35	½	
Neutral.. 170	353	134	61	228	30	198	585	77	508	39	33	6	5.0	+	34	1	*
Acid..... 34	372	140	50	270	55	215	540	110	430	50	36	14	3.9	+	23	1½	
	408	158	42	322	87	235	555	150	405	58	39	19	4.6	+	51	2	

* Bile in resting stomach.

(col. 7) was always much lower than we have ever observed in any normal dog. Out of 13 half-hour samples, the single high value was 116 mgm. of acid chloride per 100 cc. of gastric contents, the remaining samples ranged from 32 to 98 mgm. per 100 cc., and during any single experiment the

values showed only small changes. The acid chloride concentration of the total secretions entering the stomach (col. 10) was also low due to the large amount of non-acid fluid of duodenal origin.

2. *Dog X. Mann-Williamson operation with the duodenum drained into the jejunum below the gastrojejunostomy.* An interesting finding in this dog was the occasional presence of bile in both the resting stomach and during a test with the Liebig's extract meal. The amount of bile and hence the amount of non-acid fluid of duodenal origin, caused pronounced changes in the gastric acidity curve. This is well illustrated by comparing the acid chloride concentration of the total fluid entering the stomach (col. 10) in experiment I in which a copious regurgitation of duodenal contents occurred, and in experiment II in which no bile was found in the gastric sample. In experiment I the values are quite normal, while in experiment II they are extremely high and resemble the values found in whole stomach pouches (8). As was previously shown these high values are due first to the fact that the non-acid fluid of intragastric origin is small in amount, and second, to the fact that absorption of water with concentration of the acid occurs. Water absorption also causes a high total chloride concentration of the total fluid entering the stomach. In the other experiments on this dog varying amounts of bile were found and the values are intermediate between the extremes found in experiments 1 and 2.

When the non-acid fluid (col. 13) is compared in dogs V and X the difference is striking, due to the difference in the amounts of non-acid fluid of intragastric and duodenal origin as pointed out in previous publications (9, 10). When there was no evidence of water absorption, the neutral chloride non-acid fluid ratio was practically the same in the two dogs.

Discussion. Four of the dogs in which the duodenum was drained into the stomach lived from 8 to 39 weeks and if we discount the acute erosion in dog VI, as a terminal event associated with the cause of death, then none of the animals developed the typical ulcer seen after the Mann-Williamson operation. The recent statistical studies of Orndorff, Fauley and Ivy (11) have shown that following the Mann-Williamson operation 70 per cent of dogs will develop ulcer in 8 weeks and 100 per cent after 14 weeks. It is thus evident that when duodenal secretions are drained into the stomach, the jejunum is protected from ulcer. While this work was in progress a paper appeared by Graves (12) in which he reports a similar degree of protection and also ascribes the results of McCann to improper drainage of the duodenal contents into the stomach.

The gastric analyses on dog V show that although large amounts of acid were being secreted, the diluting and neutralizing effects of the duodenal secretions kept the *total* acidity of the gastric contents low, with one exception the values ranged from 32 to 98 mgm. of acid chloride per

100 cc. Since ulcer did not develop it is evident that there is a definite threshold value of acid necessary for ulcer formation in the jejunum, and that the above values are below the threshold level. The studies of Dragstedt (13) have shown that the threshold value for the digestion of living tissues lies between 0.10 and 0.15 per cent of *free* acid (97 to 146 mgm. of acid chloride per 100 cc.) which is considerably higher than the values for the *total* acid found in dog V.

The anorexia and loss of weight which occurred when the duodenum was drained into the stomach may be very profound. It appears likely that the anorexia is due to the distention of the stomach caused by the large amounts of duodenal secretions constantly present. The loss of weight, which may be as profound as that which occurs after the ordinary Mann-Williamson operation, was also noted by Graves (12) and by Ivy and Fauley (7). It cannot be explained solely by the anorexia since this was absent in dogs IV and VI. It is quite possible that it is due to the fact that certain essential substances in the duodenal secretions are destroyed by pepsin and hydrochloric acid and hence the organism is deprived of them just as when the duodenal secretions are drained into the lower ileum. Ivy (14) attributes it merely to insufficient intestinal digestion.

The frequent finding of bile in the gastric contents when the duodenum was drained into the jejunum shows that after the ordinary Mann-Williamson operation the succus entericus of the jejunum may at times regurgitate into the stomach and keep the acidity lower than it would otherwise be. This may have been a factor in the experiments of McCann (6) in which he found that there was no essential change in the acidity after operation.

SUMMARY

1. When the operation of surgical duodenal drainage was performed and the duodenum drained into the stomach in a manner which provided for adequate stimulation and free drainage the typical ulcers did not form in the jejunum. Gastric analysis showed that the total acidity of the gastric contents was definitely below the threshold value for the digestion of living tissues as determined by Dragstedt.

2. When the duodenum was drained into the jejunum below the gastrojejunostomy ulcers did not occur. Bile was often found in the gastric contents, indicating that jejunal contents may regurgitate into the stomach after operation. The acidity curve varied with the amount of duodenal regurgitation being very high when no bile was present and lower as more regurgitation occurred.

3. Marked weight loss and anorexia were frequent when the duodenal secretions were drained into the stomach but not when they were drained into the jejunum.

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THE ENDOMETRIAL VASCULAR BED IN RELATION TO RHYTHMIC UTERINE MOTILITY, WITH A CONSIDERATION OF THE FUNCTIONS OF THE INTERMITTENT CONTRACTIONS OF OESTRUS[1]

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The regulation of the changes taking place in the endometrial vascular bed has attracted the attention of a number of recent investigators (E. Allen, 1935) and has been shown to be under the control of the ovarian hormones. Recent work on skeletal and smooth muscle (Anrep, 1934–35) gives ample evidence, however, that muscular activity has a profound effect upon the volume flow of blood through muscles. No attempt has been made up to the present time to study the effect of myometrial activity upon the endometrial vascular bed which lies encased within a circle of uterine muscle. The purpose of the present paper is to report observations which deal with this subject.

In this study a correlation is made between the distribution and size of the small vessels and capillaries of the endometrium when there is 1, no uterine motility as in the absence of oestrin; 2, following the administration of oestrin but prior to the establishment of motility; and 3, also after treatment with oestrin but in the presence of marked rhythmic uterine motility.

METHODS AND MATERIALS. Twelve mature female rabbits of mixed stock were ovariectomized on the seventh day of pseudopregnancy. At this time the uteri are in comparable functional states. Nine days later uterine fistulae were made as described elsewhere (Reynolds, 1930; Reynolds and Friedman, 1930) and left untouched for another ten days, so allowing ample time for the hyperemic effects of the operation to subside. By the nineteenth day following ovariectomy the uterus is quiescent (Reynolds, 1931).

Four of the rabbits were left untreated whereas the other eight received a single injection of two hundred rat units of oestrin[2] intramuscularly on the nineteenth day. Forty-eight hours later records of motility were obtained from one uterine horn of each unanesthetized rabbit. Full anesthesia was then induced by Dial (Ciba)

[1] Aided by a grant from the Committee for Research in Problems of Sex, of the National Research Council.

[2] Progynon-B, generously supplied by the Schering Corporation. We are indebted to Dr. Erwin Schwenk for this hormone.

administered intramuscularly following which the abdomen was opened, the uterus gently exposed and the intestines packed out of the way in such a manner that there was minimal pressure on the large vessels of the mesometrium. The incision was then closed with clamps and the abdomen left untouched in order to minimize any disturbing influence of the above procedures. After a half-hour the wound was gently opened and 95 per cent alcohol, chilled with solid carbon dioxide (Dri-Ice), was poured quickly on a loop of *relaxed* uterus. The tissues froze almost instantaneously. The uterine horns used in recording motility were carefully avoided at this time, and in rabbit V-10 the uterus was contracted at the time of freezing. The solidified tissues were then excised and placed in chilled alcohol. The bottles, wrapped in cotton wool, were placed in the freezing compartment of a refrigerator and over the course of twelve to eighteen hours were thawed gradually. Throughout the next day the bottles were slowly warmed to room temperature. The tissues from the first five rabbits were cut at 12μ and stained with hemotoxylin and eosin; the tissues of the remaining seven rabbits were cut at 7μ. The results included in this report are based upon the counts made in these last seven rabbits since the thinner sections permitted very accurate studies. At least two hundred and fifty serial cross sections were obtained from each block and were studied in the manner described below.

Vascularity index. An index to the capillary and small vessel content of the endometrium was obtained in a manner similar to that described by Krogh (1929) in other tissues, except that we used no injection-mass. This technic consists of determining the number of open vessels per square millimeter in the average cross-section of endometrium. Drawings of projections of one endometrial fold were made from every tenth serial section for at least two hundred sections. The total length of uterus represented in the count was 1.4 mm. or over. It was ascertained that vessels as small as circa 4 μ diameter could be regularly identified. Extensive preliminary practice with the technic showed that counts from different drawings of the same area of tissue agreed very closely, and that the same vessels were usually included from drawing to drawing, even when these were made on different days.

When counts from untreated, ovariectomized rabbits and from oestrin-treated rabbits were made it was soon learned that a certain degree of arbitrary judgment had to be exercised to select histologically comparable areas as far as freedom from edematous and disruptive changes were concerned.

The area, expressed in square millimeters, of each section of endometrium studied was determined by a planimeter, due allowance being made for the magnification of the drawing. The number of blood vessels was counted from the drawings and the number of open vessels per square millimeter of cross-sectional area thus obtained. In order to permit adequate statistical treatment of the data, twenty-five hundred to three thousand and in two instances well over four thousand vessels were counted in the total of nineteen to twenty-four sections from the various rabbits. As stated, the counts were limited to histologically comparable areas, as regards freedom from edema.

RESULTS. Summarized in table 1, in order of increasing uterine motility, are the results from uteri fixed, prepared and studied in the manner noted above.

Endometrial vascular population. It may be seen that the highest vascular counts were found in the two untreated ovariectomized rabbits having no uterine motility (V-11 and V-12). In contrast to these are the distinctly lower counts made from uteri of oestrin-injected rabbits. The

highest of the counts in these latter rabbits was from a highly active uterus (V-6), and rabbit (V-10), whose uterus was nearly as active, gave only a slightly lower count. Rabbits V-7, V-8, and V-9, also oestrin-injected, possessed uteri which were virtually inactive at the time the tissues were taken, nevertheless counts from these uteri are comparable to the counts in the two active uteri noted above. Another rabbit (V-3) not included in table 1 had a very active uterus with such extensive edema that an estimate of the density of the vascular population was impossible.

Although the number of the small vessels per square millimeter in these endometria is but a fraction of the values quoted by Krogh (1929, pp. 28–31) for skeletal muscle, the accuracy of the two methods is comparable since the percentage dispersion, indicated in table 1 for the present data, is of the same order of magnitude in both types of tissue.

TABLE 1

Correlation of rhythmic uterine motility and the density of the endometrial vascular bed (V-11 and V-12, no oestrin; all others, 48 hours after oestrin)

RABBIT	MOTILITY	NUMBER OF SECTIONS	AVERAGE NUMBER OF VESSELS PER SQUARE MILLIMETER	STANDARD DEVIATION	LIMITS OF P.E. MEAN	PER CENT DISPERSION	EDEMA
						per cent	
V-11	0	20	229.5	±47.3	222.5–236.5	21	0
V-12	0	20	189.1	±29.6	184.7–193.5	16	0
V-7	0–+	19	106.3	±24.0	101.7–110.9	22	0
V-8	0–+	20	115.9	±11.5	114.1–117.6	10	+
V-9	+	19	92.2	±19.5	90.1– 95.1	21	+
V-6	+++	22	125.9	±18.9	125.2–128.6	15	++
V-10	++	24	114.6	±17.7	112.2–117	15	++

These results betoken, therefore, a diminution of about 50 per cent in the density of the vascular population of the endometrium after the administration of oestrin. This reduction takes place prior to the initiation of motility. As the tissues become increasingly edematous, sub-epithelial hematomata are found and under the conditions of these experiments are associated with marked uterine motility. It may be noted that the diminution in number of vessels per unit area of endometrium of active uteri as contrasted with the greater number in uninjected rabbits is opposite to the situation found in skeletal muscle. In the latter, a great increase in the number of open vessels is associated with activity (Krogh, 1929). Some of the reasons for the reverse situation in the endometrium are discussed below.

Size of the endometrial vessels. The differences in average diameter of the small vessels of the untreated and treated rabbits respectively are shown in

figures 1 and 2. It will be seen in figure 1, drawn from a section of quiescent uterus from an uninjected rabbit (V-11), that the number of very small vessels is relatively great, whereas in figure 2, drawn from tissues from an oestrin-activated uterus (V-6), it will be seen that the proportion of smaller-sized vessels is low. This is one of the outstanding features of the vascular patterns noted in these studies, namely, that a state of vascular exaltation is commonly observed in endometria of oestrin-injected rabbits. Equally important is the further observation that the state of exaltation is no greater in active uteri from oestrin-treated rabbits than it is in inactive uteri, provided oestrin has been injected. The vasodilatation is, therefore, independent of myometrial activity.

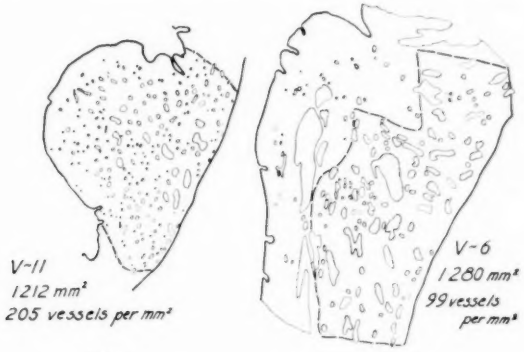

Fig. 1 Fig. 2

Fig. 1. Drawing of the vascular pattern of an endometrial fold in rabbit V-11 (originally drawn at a magnification of 131×). Rabbit ovariectomized three weeks; no uterine motility. Note the high vascular count, the small average size of the majority of vessels, and the evident closeness of the vessels.

Fig. 2. Drawing of the vascular pattern of a portion of an endometrial fold of rabbit V-6, 48 hours after the injection of oestrin. Ovariectomized for three weeks. Note the low vascular count, made in area at right since area at left was edematous. Note also the larger average size of the smallest vessels compared with figure 1, and the evident greater intervascular spaces as compared with figure 1. See the text for discussion.

Attention is also called to the more extensive intervascular areas that may be seen in oestrin-injected rabbits (fig. 2) than in the uninjected ones (fig. 1). This difference is attributable to an increase in the fluid matrix of the interstitial tissues of the endometrium and is a dominant factor in the reduction in density of the vascular population of the endometrium which follows the injection of oestrin. These changes also constribute to the well-known enlargement of the uterus that takes place under the influence of oestrin.

The vasodilating action of oestrin has been described in endometrial

transplants to the eye (Markee, 1932) and in the myometrium as observed through an abdominal window in rabbits (Pompen, 1933). It is found in these studies that an "initial blush" (Markee) or "peracute effect" (Pompen) occurs within a half-hour after the injection of oestrin. The initial hyperemia subsides within the next few hours but an appreciable degree of heightened color persists for the duration of the time the hormone is effective. The present results provide a basis upon which this diminution of the hyperemia may be explained since they show that the vessels become less dense in number per unit area of tissue owing to the increased permeability of the vessels upon dilatation, and the transudation of fluid which results therefrom.

Effect of myometrial contraction on the endometrial vessels. The uterus of rabbit V-10 was frozen when it was contracted, and because of this fact certain features observed in it are of interest. Table 1 shows that the number of the small blood vessels per square millimeter is similar to that found in the other oestrin-injected rabbits. In contrast to them, however, the average diameters of the vessels of V-10 are almost uniformly small, giving to the observer an impression like that which is obtained from uteri of untreated rabbits. In this case, therefore, the contraction has reduced the average size of the vessels. This is evidently accomplished by forcing ahead the blood which was in them when they were dilated. Such an interpretation is in accord with the observation of Anrep (1935) that the volume flow of blood in smooth and striated muscle is greatly increased by intermittent contractions, owing to the pumping action of the movements.

Discussion. These results show that an orderly change takes place in the vascularity of the endometrium following administration of oestrin to ovariectomized rabbits. Work of others as well as our own shows that a marked vasodilatation takes place. Associated with this is an increase in capillary permeability which ultimately allows the development of edema. As a result of these changes a reduction occurs in the density of the vascular population. Inasmuch as these changes precede the initiation of rhythmic uterine motility, they are independent of it. Nevertheless intermittent contractility of the myometrium is not without effect upon the endometrial vascular bed.

In the first place, it serves to increase the volume flow of blood through the dilated endometrial vessels. In this way, freedom from congestion may be assured.

The edematous changes described above suggest one further point for consideration. Powerful rhythmic uterine contractions should serve to remove escaped plasma proteins and fluid by way of the lymphatic vessels. It will be recalled that lymph flow from most organs is facilitated by and largely depends upon intermittent activity of the parts concerned (Drinker and Field, 1932). In addition, it may be further recalled that Andersen

has shown that the lymphatic vessels of the genital tract may be easily injected only at the time of oestrus (Andersen, 1927).

In view of the foregoing considerations, it appears, therefore, that two of the principal functions served by rhythmic uterine contractility during oestrus may be to effect on the one hand an increased volume flow of blood through enlarged, hyperemic vessels and on the other, to act as an effective agent for the removal of some of the edema fluid found at this time. In this manner, rhythmic uterine motility would serve to limit and repair the vascular and fluid matrix changes induced by the direct action of oestrin.

CONCLUSIONS

1. The density of the endometrial vascular population was studied in ovariectomized rabbits, some of which were untreated, others of which were injected with oestrin. The number of open small vessels per unit area of average cross section of endometrium was correlated with the degree of uterine motility in each instance.

2. In untreated ovariectomized rabbits, the motility of the uterus was nil, and the number of open small vessels was high (circa 200 per sq. mm.). The average diameters of these vessels is small.

3. After oestrin administration, the vessels become dilated and their permeability increases. The endometrium becomes edematous, and the density of the vascular population becomes less (circa 100 per sq. mm.). These changes precede any appreciable development of rhythmic motility.

4. When the uterus exhibits rhythmic motility, the vascularity is low, the tissues edematous, and the small vessels much dilated. Sub-epithelial hematomata are also present. The relation of rhythmic motility to volume flow of blood and lymph flow in the uterus is discussed.

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THE EFFECT OF CERTAIN SULFUR COMPOUNDS ON THE COAGULATION OF BLOOD

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In the course of some metabolism experiments with various sulfur-containing compounds, carried out in the laboratory of the Lankenau Hospital Research Institute, confirmation of the inhibiting effect of cysteine on coagulation reported by Mueller and Sturgis (1932) was obtained. A similar effect of methionine was observed in *in vivo* but not in *in vitro* experiments. In an attempt to explain the mechanism of this inhibiting action, the following series of experiments was undertaken.

I. *In vitro experiments with whole blood.* Cysteine hydrochloride, taurine, methionine, glycine, alanine and cysteic acid, neutralized to pH 7.0 ± 0.1, were added in graded amounts to whole blood giving final concentrations of from 4.4×10^{-5} to 0.18 *M*, and the coagulation time was determined by the 8 mm. tube method. The results are summarized in figure 1. The abscissae represent molar concentrations of the substances, 0.4 cc. of which was added to 1 cc. of whole blood. It may be seen that a marked inhibition of coagulation occurred when taurocholic acid, taurine and cysteine were employed, whereas methionine had no effect to the limit of its solubility.

II. *In vitro experiments with the isolated components of the blood clotting system.* The components of the coagulation system were isolated by the following methods: Prothrombin was prepared from horse, rabbit and human plasmas by the method of Mellanby (1931). Fibrinogen was repeatedly salted out from horse plasma with sodium chloride (Eagle, 1934–35). The tissue factor was obtained from two sources: a, platelet suspension (Eagle, 1934–35), and b, desiccated rabbit lung (Eagle, personal communication), by drying in the Flosdorf-Mudd desiccator.[1] Calcium was added as 1 per cent $CaCl_2$. The pH was controlled at 7.0 ± 0.1 with bromthymol blue as indicator. From a stock solution of cysteine hydrochloride, freshly prepared immediately before using and adjusted to pH

[1] The authors are indebted to Sharp and Dohme for generous supplies of horse blood and also to Doctor Flosdorf of the Department of Bacteriology of the University of Pennsylvania for use of their Flosdorf desiccator.

7.0 ± 0.1 with NaOH, a series of dilutions was made. In a typical experiment the following procedure and amounts of constituents were used:

Into a carefully cleaned test tube (70 mm. × 8 mm. inside diameter) were placed 0.01 cc. of $CaCl_2$ solution and approximately 2 mgm. of desiccated rabbit lung or 0.02 cc. of platelet suspension. Two-tenths cubic centimeter of prothrombin solution was added, the material thoroughly mixed by shaking the tube and allowed to stand for 10 minutes (which time was found to be adequate for maximum formation of thrombin). Eight-tenths cubic centimeter of fibrinogen solution was added, the

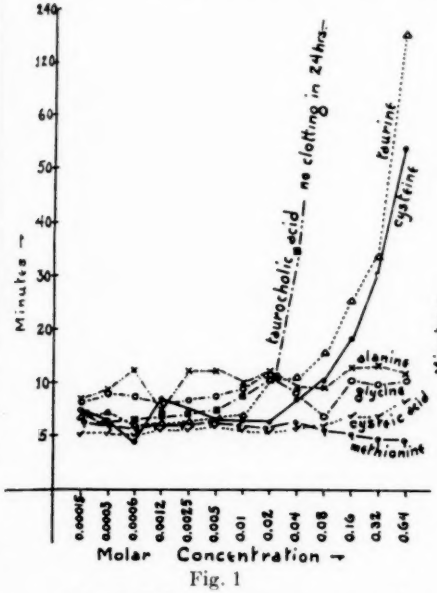

Fig. 1

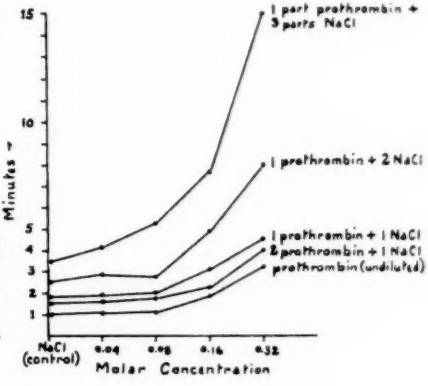

Fig. 2

Fig. 1. Effect of various compounds on the coagulation of whole blood. Abscissae, molar concentrations added, 0.4 cc. to 1 cc. of whole blood. Ordinates, time in minutes.

Fig. 2. Effect of cysteine on coagulation time with varying concentrations of prothrombin. Abscissae, molar concentrations of cysteine. Ordinates, coagulation time in minutes.

materials mixed by a few quick shakes, and the tube examined for coagulation at 10 to 15 second intervals until it could be inverted without appreciable change in the level of the contents. The coagulation time, the time elapsing between the addition of fibrinogen and the clotting, ranged with different batches of the preparation from 30 seconds to 4 minutes, with the majority around 1 minute. The addition of cysteine at different steps in the procedure will be described below. As a control, physiological salt solution (0.85 per cent NaCl) was added in similar amounts at the corresponding time.

a. *Effect of cysteine on coagulation with varying dilutions of prothrombin.* From a solution of prothrombin which gave a coagulation time of 1 minute,

varying dilutions with physiological salt solution were made. To tubes containing calcium and tissue factor, 0.4 cc. of cysteine in concentrations from 0.32 *M* to 0.04 *M* followed by 0.2 cc. of the prothrombin in a series of dilutions in physiological salt solution were added and allowed to stand for 10 minutes before the addition of fibrinogen. The coagulation times, given in figure 2, are increasingly prolonged in the tubes containing 0.16 and 0.32 *M* cysteine, with an accentuation of the curve in those tubes with high concentrations of cysteine and more dilute prothrombin solutions.

TABLE 1

Effect on coagulation time of cysteine added before and after thrombin formation

Calcium chloride sol. (1 per cent) 0.01 cc.; platelet suspension, 0.02 cc.; prothrombin sol., 0.2 cc. cysteine hydrochloride (neutralized) in varying concentrations and 0.8 cc. fibrinogen solution were employed. In *a* cysteine was added before the prothrombin, in *b* cysteine was added 10 minutes after the prothrombin.

	CONTROL	MOLAR CONC. CYSTEINE					
		0.02	0.04	0.08	0.16	0.32	0.64
a. Cysteine added before thrombin formation. Coag. time (mins.).	4	4	7	24	77	167	600
b. Cysteine added after thrombin formation. Coag. time (mins.).		4	4	4	6	7	17

TABLE 2

Effect on coagulation time of varying periods of thrombin formation in the presence of cysteine

	COAGULATION TIME									
	Period of thrombin formation (min.)									
	5	10	15	20	30	60	120	180	240	300
Control (sec.)	60	60	60	55	60	55	60			
0.04 *M* cysteine (sec.)	60	55	60	60	65	60	60			
0.16 *M* cysteine (sec.)	85	80	105	110	140	150	140			
0.32 *M* cysteine (sec.)	370	320	310	425	425	410	355			
0.64 *M* cysteine (min.)	117	216	246	180	326	180	100	155	190	215

b. *Effect of cysteine on coagulation when added at different phases of the coagulation process.* Table 1 shows the marked difference in the effect of cysteine when added at different phases of the coagulation process. If thrombin is allowed to form for 10 minutes before cysteine is added, the inhibition of coagulation is relatively slight compared with the considerable delay in clotting occasioned by the addition of cysteine before thrombin formation, i.e., when cysteine in 0.32 and 0.64 molar concentrations was added before thrombin formation, the coagulation times were 167 and 600

minutes, respectively, as against 7 and 17 minutes when the cysteine in the same concentrations was added after thrombin formation.

c. Effect on coagulation time of varying periods of thrombin formation in the presence of cysteine. Calcium, tissue factor, cysteine and prothrombin were allowed to react for varying intervals of time before the fibrinogen was added. The results, given in table 2 show that the maximal inhibition of coagulation was obtained within 5 minutes and that no additional thrombin was formed during the following 2 to 5 hours.

TABLE 3

Effect of cysteine on thrombin

Two-hundredths cubic centimeter 1 per cent $CaCl_2$, 0.4 cc. prothrombin solution and approximately 2 mgm. desiccated rabbit lung, allowed to stand 10 minutes. Four-tenths cubic centimeter of 0.64 *M* cysteine was added followed by fibrinogen after varying periods. The control tube, in which the cysteine solutions were replaced with physiological salt solution, clotted in 60 seconds.

	TIME BETWEEN ADDITION OF CYSTEINE AND FIBRINOGEN (MIN.)						
	5	10	15	20	30	60	90
Coagulation time (sec.)	210	305	335	515	570	705	645

TABLE 4

Effect of various substances on coagulation time when added to the system before and after thrombin formation

	CONTROL NaCl	MOLAR CONC.			
		0.04	0.08	0.16	0.32
a. Added before thrombin formation:					
Cysteine (sec.)	75	95	160	230	360
Ascorbic acid (sec.)	95	95	95	95	95
Phenosafranine (sec.)	120	120	125	120	120
Taurine (sec.)	90	95	95	100	95
Taurocholic acid (sec.)	85	3 hrs.			
Taurocholic acid (sec.)	85	3 hrs.			
b. Added after thrombin formation:					
Taurocholic acid (sec.)	100	450	20 min.	43 min.	76 min.

d. *Effect of cysteine on thrombin for varying periods, as indicated by coagulation time.* Thrombin was allowed to form during the usual 10 minute period. Then 0.4 cc. of 0.64 *M* cysteine was added, followed by fibrinogen solution at varying intervals from 5 to 90 minutes (table 3). The slight inhibiting effect on coagulation became constant after about 30 to 60 minutes.

e. *Effect of various other substances on coagulation before and after thrombin formation.* Ascorbic acid and phenosaphranine, two compounds with reducing properties comparable to those of cysteine, produced no effect on rate of coagulation, as seen in table 4. Taurine, added to the coagulation system set up with isolated components, had no effect, contrasting with the marked effect produced when added to whole blood. Taurocholic acid, however, showed a more powerful inhibiting action than cysteine when added before thrombin formation, no clotting taking place when 0.08 *M* or

TABLE 5

Effect of cysteine on platelets (tissue factor)

Platelets obtained from horse plasma was subjected to cysteine or cysteine and $CaCl_2$ according to the procedure below. Control tests were made similarly with physiological salt replacing the cysteine.

(a) 0.5 cc. platelets + 1.0 cc. 0.64 *M* cysteine.
(b) 0.5 cc. platelets + 0.25 cc. of 1 per cent $CaCl_2$ + 1.0 cc. 0.64 *M* cysteine.
(c) 0.5 cc. platelets + 1 cc. physiological salt solution.
(d) 0.5 cc. platelets + 0.25 cc. 1 per cent $CaCl_2$ + 1 cc. physiological salt solution.

The platelets were recovered free from cysteine and 0.02 cc. used in coagulation tests as in the routine procedure.

	PLATELETS RECOVERED FROM			
	a	b	c	d
Coagulation time (sec.)	70	75	75	65

TABLE 6

Effect of cysteine on fibrinogen and prothrombin as indicated by the coagulation time

(a) Fibrinogen and (b) prothrombin treated with cysteine and recovered. Coagulation time by the 8 mm. tube method.

	COAGULATION TIME			
	CONTROL NaCl	Molar conc. cysteine		
		0.16	0.32	0.64
Fibrinogen (sec.)	40	40	50	50
Prothrombin (sec.)	80	305	28 min.	540 min.

greater concentrations were added. It also affected the rate of clotting when added after the thrombin was formed, though the effect was far less than before its formation.

f. *Effect of cysteine on the individual components of the coagulation system.* Five-tenths cubic centimeter of platelet suspension and 1 cc. of 0.64 *M* cysteine were placed together for 15 minutes and centrifuged to recover the platelets. The latter were repeatedly washed with physiological salt solution and centrifuged until free from traces of SH. In a similar manner,

platelet suspension plus $CaCl_2$ solution was treated with cysteine and the platelets recovered. In table 5 it may be seen that platelets so treated were as effective as control platelets in producing coagulation.

Samples of fibrinogen were subjected to concentrations of cysteine from 0.16 to 0.64 *M* for 10 minutes, reprecipitated, washed free from traces of SH, and taken up in buffered physiological salt solution. The results (table 6) show no effect on the fibrinogen as indicated by the coagulation rate. No appreciable gross difference in the precipitate was detected. When prothrombin was similarly treated, however, the delay in coagulation was marked and was directly proportional to the concentrations of cysteine (table 6). There was a slightly greater yield in the control and in the lower concentrations of cysteine, but there was no apparent variance in the character of the precipitate.

A sample of rabbit plasma (citrated (0.5 per cent) blood) was added to twice the amount of 0.64 cysteine, after which the prothrombin was extracted in the manner described above. This prothrombin failed to cause coagulation in 6 hours, although the control, treated similarly with physiological salt, gave no greater yield of precipitate, but caused coagulation in the normal range of time.

As a further control prothrombin was subjected to a 0.64 *M* concentration of sodium hypophosphite, with no inhibition of the coagulation time over the control of 1 minute.

III. *In vivo experiments in human subjects.* a. *Methionine.* The effects produced by the ingestion and intravenous injection of methionine in 2 human subjects are shown in figure 3. For the intravenous administration, 1.31 gram of methionine was dissolved in 50 cc. of water, buffered to pH 7.0 + 0.1 and injected into the median basilic or cephalic veins during 2 minutes. The bleeding time was determined by the method of Ivy (Ivy, Shapiro and Melnick, 1935) and the coagulation time by the 8 mm. tube method.

In both intravenous tests, depicted for the two subjects in curves *A* and *B*, the coagulation and bleeding times were definitely prolonged, although the latter was less marked in the case of *B*. The effect on the coagulation time continued almost twice as long as on the bleeding time. Curves *C* represent the effects of ingestion of 1.31 gram of methionine and curve *D*, the effects of ingestion of 3.35 grams. Following ingestion of the smaller amount, the duration of the effect was about the same for the bleeding time as for the coagulation time. After ingestion of the 3.35 grams, the curve rose higher and was more prolonged than after the ingestion of 1.31 gram.

b. *Cysteine.* One and thirty-one hundredths gram of cysteine hydrochloride in 50 cc. of water and neutralized to about pH 7.0, was injected intravenously in one fasting subject and a similar amount ingested by a second. The results are shown in figure 4. The Ivy bleeding time was increased in

both cases, rising more abruptly and falling more quickly following injection than following ingestion. The coagulation time remained within normal limits in both instances.

At intervals during the experiment, the coagulation time was determined in the presence of varying concentrations of cysteine (table 7). The usual inhibiting effect on coagulation of added cysteine was much less marked in those cases in which blood was taken at 27 minutes after ingestion and 20 minutes after injection as shown in the table, than when taken at the beginning of the experiment or at a later period. The "buffering effect"

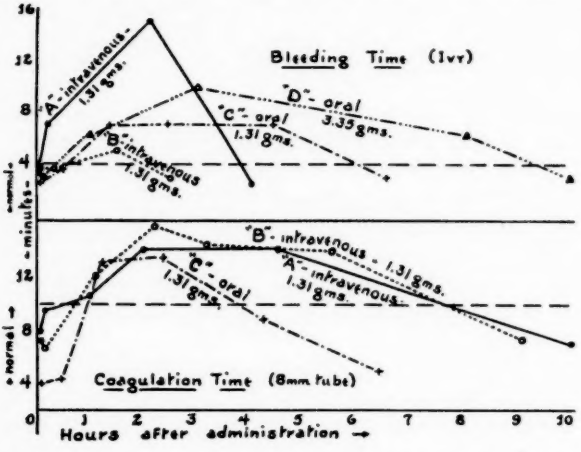

Fig. 3. Effect of ingestion and intravenous injection of methionine on bleeding time and coagulation time. Upper set of curves, Ivy bleeding time, lower set, coagulation time by the 8 mm. tube method. Curves *A* and *B*, intravenous injection of 1.31 grams methionine; *C*, ingestion of 1.31 grams; *D*, ingestion of 3.35 grams. *A* and *D* on the same subject, *B* and *C* on a second subject. Abscissae, hours after administration. Ordinates, bleeding and coagulation time, respectively.

coincided with the period of greatest prolongation of the bleeding time. (See fig. 4.)

IV. Discussion. The experimental work shows that cysteine and methionine *in vivo* and cysteine *in vitro* exert a marked effect upon the mechanism of coagulation. The effect is mainly upon only one factor of the coagulation system, prothrombin. The change in the nature of this protein is proportional to the amount of cysteine added, although no loss of SH could be detected in the supernatant liquid after reprecipitating out the prothrombin. Moreover, the effect appears to be a qualitative one, for the quantity of prothrombin recovered after addition of cysteine to a prothrombin solution, is grossly equal to that of the control precipitate. The presence or absence of the cysteine, once the nature of the prothrombin

has been affected, has little or no action on the mechanism of coagulation, since fibrinogen is unaffected and thrombin only slightly inactivated.

The nature of the action on prothrombin has not been determined. Sodium hypophosphite did not inactivate prothrombin, and ascorbic acid and phenosafranine, the latter especially having oxidation-reduction potentials closely similar to that of cysteine, produced no effect when added to the reassembled isolated components. It seems hardly probable, therefore, that the effect of cysteine is due to its reducing action. Its mode of attack is certainly different from that of taurine, which caused no delay

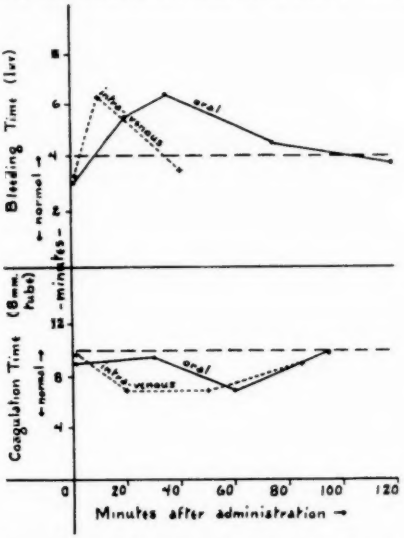

Fig. 4. Effect of ingestion and intravenous injection of cysteine on bleeding time and coagulation time. One and twenty-one hundredths grams cysteine hydrochloride (neutralized) ingested and injected in 2 different subjects. Abscissae, time in minutes after administration. Ordinates, bleeding and coagulation times, respectively.

when added similarly to the reassembled system. Taurocholic acid, on the other hand, though far more powerful in its inhibiting action, resembled cysteine in that its effect, when introduced before thrombin formation, greatly exceeded that produced when added afterwards. It is difficult to visualize any chemical action which could be possessed in common by cysteine and taurocholic acid. In view of the widespread interest recently developed in the relation of sulfhydryl to various enzyme systems and in the denaturing of proteins (Mirsky and Anson, 1934) it would be of interest to determine if a change in the number of sulfhydryl groups has occurred in the prothrombin.

These studies would confirm those of Carr and Foote suggesting that cysteine may be a responsible agent in the production of the hemorrhagic tendency in jaundice, but would indicate the defect in the formation of thrombin from prothrombin rather than in the fibrinogen factor suggested by them. In a preliminary experiment with a jaundiced patient the isolated prothrombin gave a prolonged coagulation time over a control prothrombin. But in view of the powerful inhibiting action of taurocholic acid, under conditions similar to that shown by cysteine, it would be difficult to separate these factors in jaundice.

While both the bleeding time and the coagulation time were prolonged abnormally in most instances following the administration of cysteine and methionine these deviations from normal did not always correspond.

TABLE 7

"Buffering effect" of normal blood toward coagulation-inhibiting action of cysteine: a. After ingestion of 1.31 grams of cysteine, and b, after intravenous injection of 1.31 grams cysteine

Coagulation time expressed in minutes

	CONTROL	MOLAR CONC. CYSTEINE		
		0.08	0.16	0.32
(a) After ingestion				
1 fasting	9	31	47	100
2 27 min.	9	12	16	41
3 1 hr.	7	11	21	75
4 1 hr. 36 min.	10	29	38	72
(b) After injection				
1 fasting	10	15	23	48
2 20 min.	7	8	9	30
3 50 min.	7	10	14	40
4 1 hr. 25 min.	9	15	26	54

In figure 3, experiment *B*, the bleeding time following the intravenous injection of methionine is but slightly increased, in contrast to the marked variation from normal as seen in *A* under similar conditions. The corresponding coagulation time curves in these two experiments were almost identical. The results, in figure 4, show an abnormally prolonged bleeding time. In other similar experiments, not shown, the bleeding time was similarly high, and there was also a considerable increase in the coagulation time. Ivy, Shapiro and Melnick (1935) suggest that "prolonged coagulation time in jaundice is not so much an index of bleeding tendency as of liver damage." The work reported above seems to indicate that disturbance in sulfur-amino acid metabolism, without necessarily involving liver damage, may be responsible for a delayed coagulation time as well as prolonged bleeding time.

The effect of methionine *in vivo* is in sharp contrast to its behavior *in vitro*. In experiments of the latter type, there was no delay in coagulation with final concentrations of methionine up to 0.09 *M*, whereas following the administration of methionine to the human subject, with an estimated final concentration in the blood stream of 0.0016 *M*, there was a delay in the coagulation time to 12 to 16 minutes. A bleeding time, taken 10 minutes after the intravenous injection of 1.31 gram, was almost twice the value of the upper normal limit; the coagulation time, done about the same time, was normal but reached definitely elevated values after an hour. Has there been a demethylation of the S-CH_3 grouping (i.e., production of homocysteine) to bring about the coagulation delay, or has the methionine stimulated the release or production of some other substance (i.e., cysteine) to effect the change?

SUMMARY

1. Cysteine, taurine and taurocholic acid, added to whole blood, delay coagulation.

2. The action of cysteine in inhibiting coagulation is on prothrombin, preventing activation to thrombin. Tissue factor, calcium, thrombin and fibrinogen are little or not affected by it.

3. Ascorbic acid, phenosaphranine and sodium hypophosphite do not show any inhibiting effect under the conditions of these experiments.

4. Cysteine and methionine, administered orally and intravenously in the human subject, prolong both the bleeding time and the coagulation time.

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A COMPARISON OF THREE METHODS OF MEASURING PLASMA DILUTION AFTER INTRAVENOUS SALINE INJECTION INTO NORMAL ANESTHETIZED AND FUNCTIONALLY EVISCERATED DOGS

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The problem of measuring *changes* in the plasma volume is of fundamental importance where fluid transfer within the body is involved, such as occurs in response to heat and cold, in exercise, fatigue and in hemorrhage and traumatic shock. At present the most suitable method is to use a modification of the Keith-Rowntree-Geraghty method (1916) as described by Smith (1920) in which repeated injections of a dye are made, one injection for each measurement. Objections to this method are that rapid changes of plasma volume cannot be measured since a time interval estimated by Wollheim (1928) at 40 to 80 minutes must elapse between successive measurements. With this method a sample of plasma to be used as a standard must be drawn before each dye injection and difficulties arise due to mixing time, accumulation of dye and the possibility of a variable tolerance to the dye as suggested by Lindhard (1926). An advantage in this method is that it measures the absolute plasma volume. At the present time this is perhaps the best method for measuring plasma volume changes and is superior to the methods in which concentration changes of some naturally occurring substance, as plasma protein or hemoglobin, are determined, and with the assumption that the total amount of these substances remains a constant the dilution or concentration of the plasma can be measured.

A possibility of a simple method for measuring changes in plasma volume occurs in the use of the dyes which are slowly eliminated from the vascular system. These dyes have the property of being rapidly eliminated from the vascular system during a few hours following intravenous injection but after a few days the concentration in the plasma decreases very slowly and during a two to three hour period remains practically constant. Changes in plasma volume can be determined by concentration changes of the dye. A suitable dye for this type of measurement is Wasser Blau (water soluble aniline blue), a non-toxic acid dye. This dye was found by

Wittgenstein and Krebs (1926) to persist in the plasma longer than other known dye. The elimination of this dye from dog plasma after intravenous injection has been quantitatively studied by Hemingway, Wright and Scott (1935) and it has been shown that the dye is eliminated rapidly from the plasma during the first few days following injection. About a week after injection 10 per cent of the dye remains in the plasma and is slowly excreted at a constant rate.

The purpose of the investigation reported in this paper is two-fold. In the first place the use of Wasser Blau as a dye for measurement of plasma volume changes has been investigated and, secondly, a study has been made of the ability of the vascular system to concentrate hemoglobin, plasma protein and a previously injected dye in normal and eviscerated dogs after saline injection.

From the work of Chanutin, Smith and Mendel (1924), and Calvin, Smith and Mendel (1933), it is known that after intravenous saline injections into dogs the hemoglobin is diluted and the hemoglobin returns toward the value before injection, at first rapidly and then more slowly. Two hours after injection the blood dilution lies between 0 and +20 per cent in comparison with the value before injection. The method of saline injection has been used in the experiments reported in this paper to cause changes in plasma volume. The dilution of the plasma has been measured by noting the concentration of hemoglobin, plasma protein and Wasser Blau injected one week previously. Since it is believed that the mechanism for the storing of plasma and red cells in the vascular system is in the viscera, the same experiments have been repeated on functionally eviscerated dogs.

EXPERIMENTAL METHODS. Fasting healthy dogs were anesthetized with nembutal, 35 mgm. per kilo being given to the normal dogs and 40 mgm. per kilo being given to dogs which were to be functionally eviscerated. The dogs were placed on a heated table and their rectal temperature maintained at a constant level. About one hour after receiving the anesthetic or one-half hour after the evisceration, 0.9 per cent NaCl solution heated to body temperature was allowed to flow by gravity through a needle inserted into the femoral vein. Samples of blood were withdrawn from the femoral artery at 5, 10, 15, 20, 30, 45, 60, 90 and 120 minutes after the injection period. After withdrawal about 0.25 cc. of blood was placed in a tube containing dry potassium oxalate for the hemoglobin determination and the remaining 4 to 5 cc. were discharged into 1 cc. of isosmotic potassium oxalate in a 15 cc. calibrated centrifuge tube. The tubes were centrifuged and the cell volume and total volume determined. With the operated dogs the celiac, superior mesenteric, inferior mesenteric, renal arteries and the portal and renal veins were ligated. The viscera, including the stomach, intestine, spleen, pancreas and kidneys, were removed.

Evisceration of this type has been called functional evisceration by Peterson (1934) and is applied to that type of evisceration in which all of the abdominal viscera except the liver are removed. The hepatic artery and portal vein are ligated. The operation lasted from one-half to one hour. About one and a half to two hours after giving the anesthetic the saline was injected into the operated dogs and the same procedure followed as for the unoperated dogs except that the saline injected was equal to two-thirds of the blood volume for the functionally eviscerated dogs, the unoperated dogs receiving an amount of saline equal to the blood volume.

Hemoglobin. The relative hemoglobin concentration was determined by drawing about 0.1 cc. of the blood from the tube which previously contained the potassium oxalate into a Scott (1917) pipette, i.e., a pipette with a two-way stopcock in the middle. After drawing a sample into the pipette the stopcock was turned through 180° and the blood washed from the pipette by 0.1 *N* HCl. The acidulated blood was diluted to 25 cc. and placed in a darkened bottle for 12 hours and then compared in a Duboscq colorimeter.

Plasma protein. About one-third of a cubic centimeter of oxalated plasma was drawn into a calibrated Scott pipette and washed from the pipette by about 5 cc. of distilled water into a 50 cc. centrifuge tube. The plasma proteins were precipitated by the tungstic acid reagent of Folin and Wu, centrifuged and the supernatant fluid containing the non-protein nitrogen decanted off. Concentrated sulphuric acid containing $CuSO_4$ was added and the proteins digested in the centrifuge tube. After digestion the digest was made alkaline and the ammonia distilled through a micro-Kjeldahl distilling apparatus, as described by Cavett (1931). The nitrogen was then determined by titration by the usual Kjeldahl method.

Wasser Blau. To 1 cc. of oxalated plasma was added 1 cc. 0.2 *N* HCl, which caused the development of the blue color of the dye in previously colorless plasma. The relative dye concentrations were determined by comparison in a Duboscq microcolorimeter using the red filter as previousy described. The dye was injected into the animal one week before the experiment.

Calculation of dilution. In comparing the dilutions as measured by the hemoglobin, the value of the hemoglobin before saline injection was taken as standard and the relative plasma volume given the value of 100. The succeeding values from blood samples taken after injection of the saline were calculated from the formula

$$\text{relative volume} = 100\,\frac{l_n}{l_s}$$

where l_s is the colorimeter reading of the standard and l_n the colorimeter reading of a subsequent sample.

The relative dilution of the plasma as computed from the protein dilution was computed from the formula

$$\text{relative volume (standard 100)} = 100\,\frac{P_n\,(P_s - 1)\,(m - n)_s}{P_s\,(P_n - 1)\,(m - n)_n}$$

where P_s, P_n are the oxalated plasma volumes of the centrifuged blood sample, i.e., the true plasma volumes plus 1 cc. of isosmotic oxalate, of the standard and a subsequent sample. $(m - n)_s$ is the difference between the titration values of the blank m and the sample for the standard and $(m - n)_n$ is a corresponding difference for a subsequent sample.

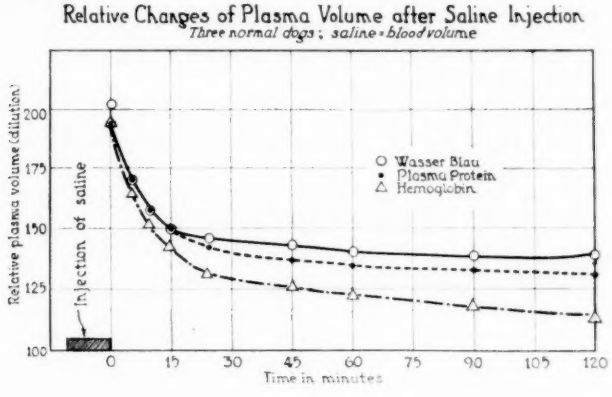

Fig. 1

The plasma volume as determined by changes in dye concentration is

$$\text{relative volume} = 100\,\frac{P_n(P_s - 1)}{P_s\,(P_n - 1)} \cdot \frac{1_n}{1_s}$$

where P_n and P_s are oxalated plasma volumes and l_s and l_n are colorimeter readings of the standard and subsequent samples.

Results. The results are given graphically in figures 1 to 3. Five minutes after an injection of saline equal to the blood volume into normal dogs anesthetized with nembutal there is a dilution of the plasma constituents of about 75 per cent (fig. 1). There is a rapid return to normal during the first 40 minutes after the injection period. During the period of from 40 to 120 minutes after injection the concentrating of the constituents is more gradual. The hemoglobin is concentrated faster than the plasma proteins while the concentrating of the dye is less than either the proteins or the hemoglobin. Two hours after the injection the blood

remains diluted as indicated by the three methods, the average dilution values being as follows: 1, hemoglobin method—15 per cent; plasma protein method—30 per cent; 3, Wasser Blau method—40 per cent. The dilution as shown by hemoglobin agrees with the results of Mendel and co-workers.

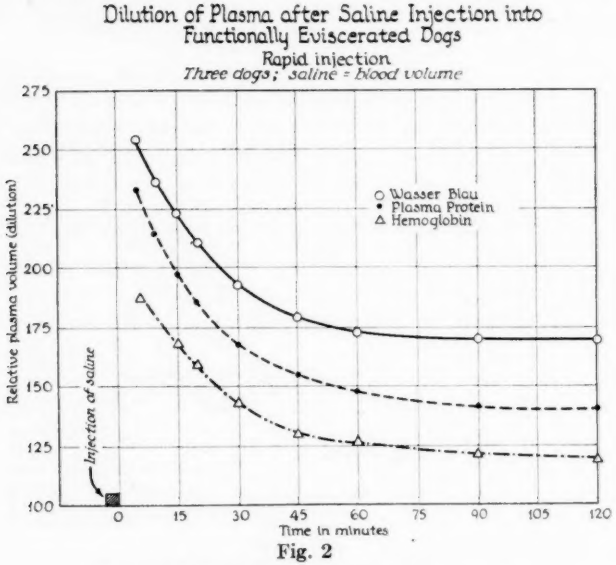

Fig. 2

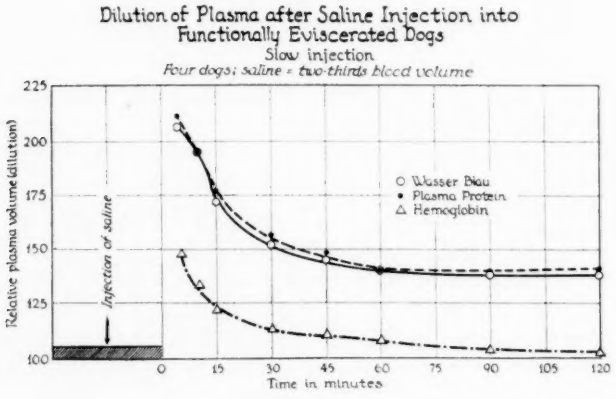

Fig. 3

In figure 2 are given the dilution values of the blood plasma after a rapid injection of saline equal to two-thirds of the blood volume into functionally eviscerated dogs. Immediately after injection the plasma dilution is

relatively great in comparison to dogs with intact viscera. In spite of the evisceration, however, the return of the plasma constituents to the normal values is rapid in the first 40 minutes and more slowly during the following 80 minutes. The dilution of the dye is greater than the dilution of the hemoglobin and the dilution of plasma proteins lies between the hemoglobin and protein. Two hours after injection the plasma volume as indicated by the Wasser Blau dilution is 70 per cent, by the plasma protein is 40 per cent and by the hemoglobin is 20 per cent.

With functionally eviscerated dogs it was expected that the dilution values of hemoglobin, plasma protein and dye would be the same after saline injection. This supposition was based on the conclusions of earlier workers who attributed to the visceral organs the property of removing or adding red cells and plasma to the circulation. The results as given in figure 2 were somewhat surprising, and it was believed that the lack of agreement between the three methods might have been due to the rapid injection of the saline, placing a strain on the vascular system by which the usual concentrating mechanisms were impaired, and protein and dye might have been lost from the blood. For these reasons it was decided to repeat the experiments using a longer injection period. Figure 3 shows the results of an injection of two-thirds of the blood volume into functionally eviscerated dogs, the injection period lasting thirty minutes. It is to be noted that after slow injection of saline the plasma protein and dye are concentrated at the same rate while the hemoglobin is concentrated faster.

Several other similar experiments were made mainly for control purposes. With a functionally eviscerated dog injection of one-twentieth of the blood volume causes a dilution of 10 to 20 per cent, which remains unchanged throughout a two-hour period. With normal dogs the concentration after slower injection resembles the concentration as indicated by the latter parts of curves of figure 1.

Discussion. These experiments are of value in establishing the part played by the viscera in experimental hydremia. The following observations from the curves are worthy of notice: 1. Immediately after saline injection the blood of eviscerated animals is diluted much more than normal animals. 2. The return toward normal concentration is rapid in both normal and eviscerated animals, the general curvature of the time-dilution curves being much the same. 3. Two hours after injection the dilution of the blood of eviscerated animals is much greater than the normal animals. The viscera act as a sort of buffer to an inflow of saline. The presence of the viscera does not affect the rate of the concentrating process but does decrease the absolute dilution.

Ludwig (1932) has postulated the existence of certain "plasma depots" in the viscera wherein the plasma can be stored out of the active circulation. Chanutin, Smith and Mendel (1924) state that the viscera play a

major rôle in water storage. Roberts and Crandall (1933) have given evidence that the portal system of the liver can remove blood from active circulation. The evidence obtained from the results given in this paper supports the view that diluted blood may be stored in the viscera but the concentrating process is a property of other tissues as well as those of the viscera.

After dilution of the plasma by saline injection into dogs with intact viscera (fig. 1) the hemoglobin returns to normal concentration faster than the proteins or the dye. For these dogs a possible explanation is that additional red cells are furnished by the spleen when the blood is diluted, causing the hemoglobin concentration to rise faster than the protein or dye concentration. The protein concentration shows a gradual return to a higher concentration during the second hour after injection while the dye concentration remains unchanged. It is possible in this case that a small amount of protein is added to the diluted plasma in an effort to restore the original concentration since it is known that protein can be rapidly formed.

The mechanism for the addition of red cells and protein supposedly lies in the viscera. With the viscera removed the concentration changes of the three constituents should be the same, that is, the curves should coincide. Figures 2 and 3 show that this does not occur. After saline injection into eviscerated dogs the dilution as shown by the hemoglobin is less and the return to a normal concentration is faster than for the plasma proteins and the dye, a result similar to that for animals with intact viscera. The lack of coincidence for the three dilution curves may be explained in two ways, namely: 1, there is a store of red cells in the bone marrow or in some other tissue which supplies red cells to the diluted blood, or 2, protein and dye leave the plasma when the blood is diluted. It is not possible to predict from the results given here which method is the effective one.

With a slow injection of saline into eviscerated and normal dogs the plasma protein changes follow closely the dye concentration changes. This suggests the formation of some sort of a molecular complex formation between the dye and protein. If the dye is bound to certain of the protein molecules the protein dilution should be the same as the dye dilution. Other evidence of the existence of such a complex is that protein precipitants which remove protein from plasma also remove the dye with the precipitated protein. This view has already been proposed by Smith (1925) who noted a parallelism between dye and protein concentration of the lymph from various parts of the body. With a rapid injection of saline into eviscerated dogs the dye dilution is greater than the protein dilution. In this case it is possible that relatively more dye than protein leaves the vascular system under the strain of the rapid injection.

Due to the uncertainty in regard to red cell reservoirs in the bone marrow and other tissues the hemoglobin changes in functionally eviscerated dogs

do not necessarily represent plasma dilutions. A better measure of plasma dilution is the protein concentration change. If it be assumed that the plasma volume varies inversely as the protein concentration after slow saline injection into normal and functionally eviscerated dogs then the dye concentration is a measure of the plasma dilution since this follows the protein concentration. The dye method, however, is not recommended for measuring rapid changes in plasma volume (fig. 3) since the dye concentration does not coincide with the protein concentration. Under usual physiological changes, however, the plasma volume changes would be of a rapidity and magnitude much less than those occurring in a rapid injection. For measuring plasma volume changes the dye method is recommended as being more convenient to use than the plasma protein method and the dye is not a natural product like the plasma protein to be added to the plasma when diluted.

The plasma volume referred to here is the total plasma volume which undergoes dilution when fluid is transferred to the plasma. This is not the "circulating plasma volume" since it has been shown by Roberts and Crandall (1933) that it is possible to have an increased total plasma volume after saline injection, without a corresponding increase in the "circulating plasma volume."

SUMMARY AND CONCLUSIONS

An amount of saline equal to the blood volume has been injected intravenously into dogs anesthetized with nembutal. The concentration changes of hemoglobin, plasma proteins and the dye Wasser Blau, injected 7 days previously, have been followed in a two-hour interval after the injection. The hemoglobin showed the least dilution after injection and returned to a dilution about 15 per cent above normal after two hours. The dye concentration and the protein concentration changes were the same in the first half hour after injection but in the latter part of the post-injection period the protein concentration increased more rapidly than the dye, perhaps due to the addition of protein to the diluted blood.

An amount of saline equal to two-thirds of the blood volume was injected slowly (during 30 min.) and rapidly (during 4 min.) into functionally eviscerated anesthetized dogs. The dilution changes as indicated by the dye and plasma proteins were the same after a slow injection but this dilution was much greater than indicated by the hemoglobin. This may indicate a storage of red cells in tissues other than the viscera. After a rapid injection the plasma dilution as indicated by the dye was the greatest and hemoglobin the least with a return to lower dilutions in the same order.

In all three sets of experiments the dilution as indicated by the dye and protein was greater than the hemoglobin. This result was unexpected for

functionally eviscerated dogs and it indicates that one or both methods for measuring plasma dilution are unreliable. Red cells may accumulate in some non-visceral reservoir or the plasma protein may leave the vascular system. If the plasma protein concentration in functionally eviscerated dogs be assumed to vary inversely as the total plasma volume, then, since the dye concentration changes coincide with the protein concentration changes, the previously injected dye method is a simple and convenient method for measuring changes in plasma volume.

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THE SPECIFIC GRAVITY OF THE BLOOD OF PIGEONS IN THE QUIET STATE AND DURING EMOTIONAL EXCITEMENT

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The physical and chemical changes in the blood of birds have been little studied. In this investigation we have measured the specific gravity of the blood of domestic pigeons in the quiet and during the excited state.

METHODS. The blood was obtained from a needle puncture of a wing vein of a pigeon in the quiet state and its specific gravity measured by the falling drop method of Barbour and Hamilton (1926). The pigeon was then excited by being restrained and teased for approximately three minutes by stimulation with a weak Faradic current. Blood was again obtained immediately thereafter from a wing vein and its specific gravity determined. The second measurement was made approximately 12 minutes after the first. Our specific gravity measurements are averages of 2 determinations in each case.

A uniform procedure was carried out in all of these experiments. Care was taken not to excite the pigeon before making the measurement in the quiet state in each one of the 28 observations. Each pigeon showed the typical signs of sympathetic stimulation during emotional excitement.

RESULTS. There was an increase in the specific gravity of the blood in each one of the 28 tests. Table 1 shows the results in ten of the observations.

The maximum, minimum, and mean values of all 28 tests were as follows: the specific gravity in the quiet state ranged from 1.0491 to 1.0538, averaging 1.0524. In the excited state it ranged from 1.0524 to 1.0555, averaging 1.0548. The increase in the specific gravity ranged from 0.0015 to 0.0036 and averaged 0.0024.

DISCUSSION. The specific gravity increase in the blood of these pigeons during excitement was decidedly less than found under similar conditions in rabbits and cats. Nice and Katz (1935) with 20 observations on rabbits found the average specific gravity to be 1.04225 in the quiet state and 1.04890 during excitement or an average increase of 0.00665; and for 10 determinations on cats 1.03985 in the quiet state and 1.04580 during excite-

ment or an increase of 0.00595. These contrast with the average increase of 0.00240 in our pigeons.

An explanation for the increase in the specific gravity of the blood during emotional excitement is found as follows. The combined factors of a slight shift of water from the plasma into the tissues, Nice and Katz (1934); the inpouring of blood cells into the general circulation, Binet (1927); Barcroft (1930); and the addition of the products of tissue metabolism into the circulating blood causing an increase in the sugar, urea, uric acid, total and preformed creatinin, cholesterol and hemoglobin contribute to the augmentation in the specific gravity during emotional states, Nice and Katz (1934) and (1935).

TABLE 1

The specific gravity of the blood of pigeons

EXPERIMENT NUMBER	QUIET STATE	EXCITED STATE	INCREASE
1	1.0528	1.0553	0.0025
2	1.0524	1.0548	0.0024
3	1.0528	1.0553	0.0025
4	1.0526	1.0543	0.0017
5	1.0524	1.0553	0.0029
6	1.0491	1.0524	0.0033
7	1.0535	1.0553	0.0018
8	1.0524	1.0550	0.0026
9	1.0528	1.0548	0.0020
10	1.0524	1.0553	0.0029
Average.....	1.0523	1.0548	0.0025

SUMMARY

The specific gravity of the blood in each one of our 28 observations on pigeons showed an increase during emotional excitement. This increase was decidedly less than previously found under a similar condition in cats and rabbits.

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ACTION AND EXCITABILITY IN MAMMALIAN A FIBERS

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The activity ensuing when a nerve is stimulated is made up of a chain of processes giving electrical, potential signs. The algebraic sum of these potentials is called the "action-potential." Step by step the latter term has become more comprehensive in meaning, and as the subject has developed, other terms relating to potential have been coined to denote the phenomena observed. In the interests of clarity these terms now stand in need of correlation.

Originally the term "action-potential" was used to refer to what is now called the "spike-potential." When it was realized that the process which follows the spike varies independently of it, the term "negative after-potential" was selected to differentiate the second process from the first. Finally, in line with this terminology, the positive potential, which has long been known to come at the end of activity, was called the "positive after-potential."

For descriptive purposes a general term is needed to designate the whole sequence, and special terms are required for reference to the individual events. In accord with this need, the term "action-potential" will be used in the present paper in the comprehensive sense; and the terms "spike-potential," "negative after-potential," and "positive after-potential" will be employed for particular reference to the special features of the potential accompaniment of activity. In addition, it is often convenient to have a still more comprehensive term for the sum of the events occurring in active nerve as opposed to resting nerve, without special reference to the electrical manifestations. The expression "the action" will serve very well for that purpose.

The action-potential in all kinds of fibers, in both frog and mammalian nerves, is made up of the three components mentioned above; but there are quantitative differences. In this paper, mammalian A fibers will be described and placed in comparison with previously described frog A fibers.

Frog A fibers display in a single action first the spike, then a negative after-potential starting while the spike is in progress, and finally the positive after-potential. The negative after-potential is variable in magnitude and duration, and the positive potential which appears after the ending of

the negative after-potential is very small and may be absent. Mammalian A fibers, on the other hand, present a quite different picture, largely because of the nature of the positive after-potential. The latter is sharply defined, constant in its occurrence, and in form and position varies but little from experiment to experiment, even when special precautions for the maintenance of constancy are not observed. It appears first as a well defined trough cutting into the negative after-potential, and the action-potential is thereby given a characteristic contour. What this contour is, and the possible ways in which it may be explained by potentials of component processes added together algebraically, will now be set forth in detail.

The spike-potential. The whole train of events occurring in the action is initiated by the spike process. In order to get the form of the latter, the best procedure is to record spike-potentials in single fibers. When this is done, a result is obtained that necessitates some revision of the duration, 0.6 msec., which was given following the original observations of the mammalian spike with the cathode ray oscillograph. The value mentioned was obtained in experiments in which a lead was taken from the stimulating cathode. In that way, error arising from temporal dispersion was eliminated (except for a negligible error arising from conduction from one side to the other of the silver wire which served as the electrode (Blair and Erlanger, 1933, p. 531)); but the utilization period of the shock was included with the spike duration, and the method was such as to measure the longest components—if there be variation in the duration of mammalian A spikes, similar to that described by Blair and Erlanger (1933) for frog fibers. Records of single spikes show that for one or the other of the foregoing reasons, 0.6 msec. is too large a value.

For single-fiber records, spinal roots of the cat were used. This preparation has two advantages, both attributable to the absence of a sheath. Small strands may be separated out, so that the potential in an active fiber is shunted by only a small number of inactive fibers; and the shock artifact is reduced to a minimum. Stimuli applied at threshold cause the most irritable fiber in the nerve to respond in isolation in a percentage of trials sufficient to permit obtaining numerous single responses in a series of photographs. Records made in this way show regularly that the spike duration—and by this is meant the major part of the spike, exclusive of the tail—is very close to 0.4 msec. at 37.5 degrees C. (fig. 1). Readings of 0.4 to 0.43 msec. are obtained in the best preparations, there being uncertainty as to the precise figure, because of the impossibility of selecting precise points between which to measure. Of this duration, the period of rising potential takes up one-third, and the falling potential two-thirds of the time.

On the basis of this value it is interesting to compare the wave lengths in

roots of the cat and the bullfrog. The velocity in a threshold fiber in the cat would be 90 m.p.s., and the wave length, therefore, 3.6 cm. In the bullfrog the velocity would be 40 m.p.s., with a corresponding spike duration of 0.9 msec., giving again a wave length of 3.6 cm.

The after-potentials were observed usually on the saphenous or the phrenic nerve of the cat. Other nerves were examined only to prove that those named are typical of mammalian A fibers in general. Temporal dispersion, unless the spike height is being compared with the magnitude of the after-potentials, is not important in after-potential studies. Therefore about two centimeters of conduction were allowed to keep the action-potential conveniently free from the shock artifact. When the correct height of the spike was important, the lead was made close to the cathode.

For after-potentials, a direct current amplifier was used. The direct current amplifier is being improved continually in the interest of smaller distributed capacity, smaller drift, and a lower noise level. That used in this investigation is much better than the one mentioned in a previous paper (Gasser, 1935), but falls short of the newest model. Its calibration curve, given in figure 2, still shows somewhat more capacity effect than the A. C. amplifier, and therefore A. C. amplification was used for the measurement of spikes. The nerves were mounted in a moist chamber at 37 degrees C. and supplied with oxygen, or in some cases with oxygen containing 5 per cent of carbon dioxide.

The negative after-potential, when first visible, is completely fused with the spike. No suggestion of a rising phase, such as is often encountered in frog nerve (Gasser and Graham, 1932) is seen; and in nerves successfully rendered monophasic by the application of cocaine at the indifferent lead, spike and after-potential fuse without evidence of discontinuity (fig. 5 a). That there is discontinuity, however, can readily be brought out by painting the nerve with 1:250,000 veratrine. Without change in the spike, the negative after-potential begins to increase in size, principally by being prolonged. Starting at the junction of the spike and the after-potential, the action-potential subsides more slowly in each successive record as veratrinization progresses. Thus, when successive records are superimposed, the negative after-potentials appear to pivot about the point at which they differentiate from the spike (fig. 3).

Veratrinization also demonstrates that the decremental form of the negative after-potential is not a necessary quality of the process. In analogy with mammalian C fibers and frog A fibers, the negative after-potential should have a rising phase of its own and appear with a convex contour. Both of these qualities appear when the nerve is veratrinized. As veratrinization proceeds, rising phases develop spontaneously, especially if the nerve is in an atmosphere containing 5 per cent of CO_2, instead of in pure oxygen. But to bring out the development strikingly, the

response should be set up during the positive after-potential following a tetanus, in imitation of the procedure which has been found to bring it out in frog fibers (Gasser and Graham, 1932). The inset in figure 3 shows a growing after-potential set up in this way.

Because of overlapping with the spike, no definite value can be given for the magnitude of the negative after-potential. At the point where the slope in the decline of the action-potential begins sharply to decrease—that is, where the after-potential begins definitely to contribute to the sum of the potentials—the potential of the action is about 5–6 per cent of that obtaining at the crest of the spike. From this point the potential declines along a decremental curve. It reaches zero normally at about 15 msec. and is then continued by a deflection on the positive side of zero, which reaches a maximum at about 30 msec. and continues positive to about 70 msec. These durations are based on experiments which will be described in the latter part of this paper. Isolated nerves mounted in air show potentials which follow the same course, but the cycle is faster, so that zero potential may be reached at 12 msec. instead of at 15 to 18 msec.

Compared with the potential of the spike, the magnitude of the positive after-potential is small. When the latter reaches its maximum, a representative value would be 20 to 25 μv. The corresponding spike would reach 12 mv. at its crest; hence the after-potential amounts to only about 0.2 per cent of the spike-potential. Variations from 0.1 per cent to 0.4 per cent are encountered.

In isolated nerves the action-potential may end with the positive dip, but that mode of ending does not describe all the cases seen. There is often a suggestion of ensuing negativity. In order to describe this negativity, it is necessary to divide nerves into two groups: those which are rhythmic and those which are non-rhythmic. Nerves freshly mounted in air or oxygen—particularly phrenic nerves—in addition to the negative and positive phases already described, are apt to show a second negative potential, and even a third and a fourth one. In other words, the play between negativity and positivity behaves like a damped oscillation (fig. 4). Preparations which show this rhythmicity well are also subject to spontaneous discharging in their fibers, and this discharge increases during the negative phases and decreases during the positive ones. Such a variation in the discharge would by itself produce the appearance of rhythmicity, and hence the question is raised as to the extent to which the discharge is the cause of the rhythm. To this question the most probable answer is that the rhythm is attributable primarily to the original action. On this basis, the changes in the spontaneous discharge are readily explained: the increase by the supernormality which goes with negative after-potential, and the decrease by the subnormality which goes with positive after-potential; and without the assumption that the after-potential is rhythmic,

the variation in the spontaneous discharge would not be accounted for. Further difficulty would then also be presented in that many of the negative waves do not look like pure spike aggregates, and that often there are not enough spikes in the negative phases to permit accounting for the negativity as negative after-potential connected with the fresh discharge. On the other hand, even though the rhythm is initiated by the original action, it must be augmented and perpetuated by the resulting effect on the discharge. The bursts of spikes at the negative crests would accumulate a tendency for the potential to turn to positive again. In the negativity following this positivity, discharge would again occur, and so the cycle would continue.

The non-rhythmic group is made up of nerves in a state approximating more closely to normal, although it may fall short of giving a faithful representation of normality. Spontaneous discharge is minimal or absent. At the end of the positive trough the level of negativity is so small that one would doubt its presence, were it not that extrapolation backward from a tetanus to a single response, as the limiting case, gives support to the interpretation that the appearance of negativity is genuine (fig. 5).

After-potentials following a tetanus. After a tetanus, the negative and positive after-potentials are accentuated, particularly the latter (fig. 5). Owing to the increased tendency toward positivity, which develops during a tetanus (Gasser, 1935), the duration of the negative after-potential following the last spike in the train is curtailed, and the positive notch which succeeds it is deepened. After the positive notch, however, a very definite period of negative after-potential may appear. The latter in turn is succeeded by a second positive period.

Further development of the second appearance of negative and positive after-potential may be achieved by increasing the duration and frequency of the conditioning tetani. A concrete example will give an idea of what happens.

A nerve was tetanized for 2.2 sec. at 300 per sec., a frequency which the nerve was able to carry with full-sized spike production. At the end of the tetanus the potential dropped to + 146μv; 38 msec. after the end of the tetanus, the potential again crossed zero and reached a negative maximum of 38 μv at 75 msec.; after 425 msec. it again became positive, and remained so to the end of the record which was 3 sec. later. A result qualitatively similar, but following a shorter tetanus, is shown in figure 6 a.

Freshly isolated nerves, which show rhythmicity in the after-potentials in a single response, also show it in the after-potentials following a tetanus. Corresponding with the post-tetanic augmentation of the after-potentials, the oscillations become more definite; and it also becomes clear that the base line about which the oscillation is taking place is not the line of zero potential, but one determined by the trend of the potential resulting from

Fig. 1

Fig. 2

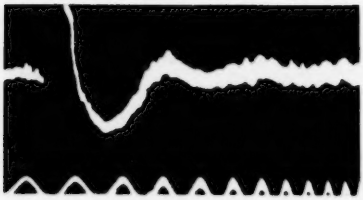


Fig. 4

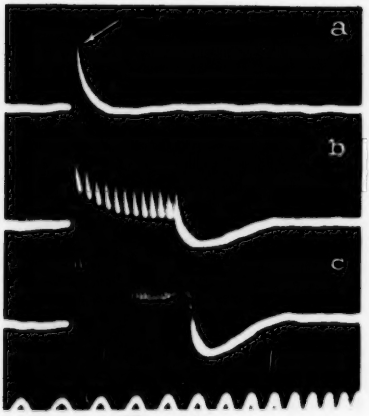


Fig. 5

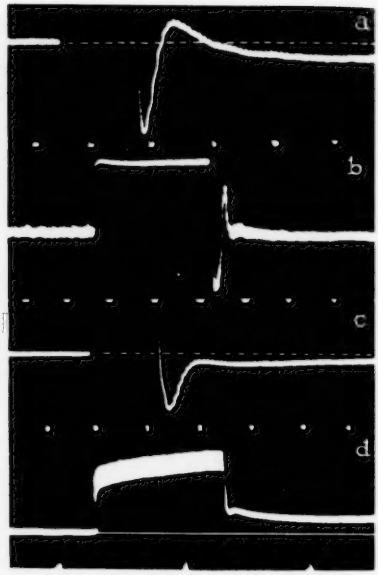


Fig. 6

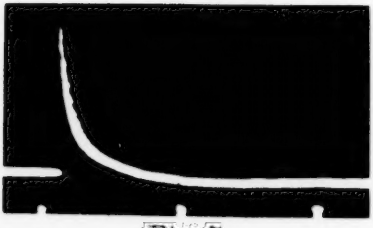


Fig. 7

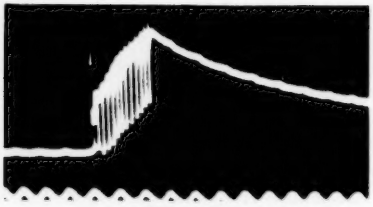


Fig. 8

Figs. 1, 2, 4, 8

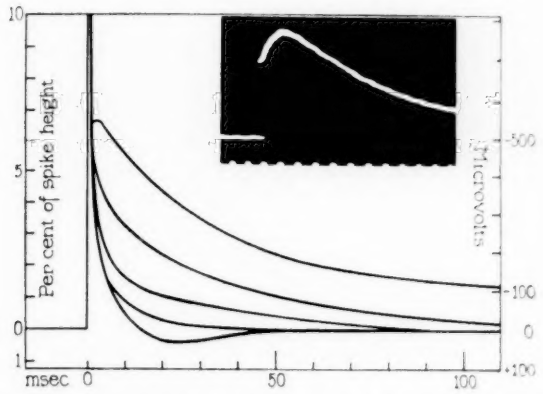


Fig. 3. Successive stages in the development of the negative after-potential in a veratrinized phrenic nerve. The first 4 curves are taken 0, 10, 18, and 28 minutes respectively after application of the drug. The top curve was taken after the nerve had been tetanized. Inset. Record of a single action-potential of a veratrinized phrenic nerve, set up soon after a tetanus. The spike is not visible. Time: 60 cycles.

the tetanus. The first oscillation is sharply negative (fig. 6 b); the second, however, comes near the transition to the positive potential, and while the oscillation is clean-cut it may attain little negativity even at its crest, and

Fig. 1. Form of mammalian spike recorded from a single fiber in a lumbar dorsal root of the cat. The spike is preceded by a shock artifact. Time: 0.2 msec. Temperature: 37.5°C.

Fig. 2. Calibration curve of D. C. amplifier. Time: 1 msec.

Fig. 4. After-potentials in a single action of a fresh phrenic nerve. The action starts at the break in the line and becomes visible in the record during the first negative after-potential. The small irregularities in the line are caused by spontaneous discharges. Time: 40 msec.

Fig. 5. After-potentials of a phrenic nerve of a cat rendered monophasic with cocaine. a, single response. The record starts at the junction of the spike and negative after-potential. If there had been a diphasic artifact, it would have appeared as a very sharp incisure at the position indicated by the arrow. b, c, tetani at two frequencies. Time: 20 msec.

Fig. 6. After-potential forms seen following a tetanus. The records show in succession the resting potential, the tetanus (with the bottoms of the curtailed negative after-potentials visible in parts b and d), and the sequence of events at the end of the tetanus. a, 0.25 sec. tetanus at 500 per sec. The first positive potential lasts 57 msec. and reaches 400μ volts. The succeeding negativity lasts 200 msec. and reaches 90μ volts. The second positive potential maximum is 47μv.; time: 0.2 sec. b, oscillating after-potential, time: 0.2 sec.; c, time: 0.2 sec.; d, time: 1 sec.

Fig. 7. Negative and positive after-potentials in a single response of a veratrinized phrenic nerve. Time: 1 sec.

Fig. 8. Tetanus of a veratrinized nerve. During the tetanus the heavy line is produced by the staircasing of the negative after-potentials. The vertical lines below the heavy line are diphasic artifacts. Time: 60 cycles.

the whole period of the oscillation may take place on the positive side of zero.

In order to obtain information about how long the terminal positive phase lasts in the action-potentials of tetanized isolated nerves, a few experiments were performed with the aid of a string galvanometer coupled with a one-stage D. C. amplifier. The amplifier which was very free from drift was built by Doctor Toennies and will be described in another connection. The nerves were mounted in the usual way and examined first with the oscillograph, in order to ascertain the proper strength of stimulation and to prove that the nerves were responding in a normal manner. The connections were then transferred to the galvanometer.

Because of its gradual ending, a definite figure cannot be given for the duration of the positive potential. No matter how long a period of time was allowed between readings, there was always some drift of the resting potential (amplifier drift was not a limiting factor); and when the nerves were fresh and the after-potentials at their best for study, sudden shifts of the string, probably connected with spontaneous firing in the nerve fibers, entered into the experiments as added complications. The durations given are times, previous to the final mergence of the potential with the changes which obscure it, at which positivity is still definitely discernible.

As is true in frog nerve, the degree of positivity does not increase in proportion to the severity of the tetanus; the positivity soon approaches a maximum, after which a further increase in tetanization expresses itself, not by an augmentation of the positivity, but by an increase of its duration. Following a 10 sec. tetanus, a representative figure for the positivity at its maximum would be 0.6–0.7 mv. This potential falls off gradually, reaching half relaxation within 15 to 30 sec. and approaching the end of visibility only after one to two minutes. After a 30 sec. tetanus, half relaxation requires more than one minute and extinction more than four minutes.

Variations in the magnitude of the parts of the sequence of potential changes occur, depending upon the preparation and the frequency of the conditioning tetanus. They rest in the last analysis on the ratio of negative to positive potential. When the negative component is small, the potential following the positive notch does not quite reach zero and a maximum occurs while the potential is still positive (fig. 6 c). Following the maximum, the positivity again increases slightly, then slowly falls off to zero, forming a section of the cycle comparable to the second positive period which results when real negativity intervenes (fig. 6 a). In this form of the cycle the after-potentials follow a sequence resembling one which has been seen in some frog nerves (Gasser, 1935, fig. 15). The positivity appears in the two parts which in frog nerve were labelled arbitrarily P_1 and P_2 (first and second positive components).

When the negative after-potential is large, the first positive wave is

carried up on the negative after-potential, so that the bottom of its trough appears on the negative side of zero (fig. 6 d). Between the two extremes all gradations occur. Among the numerous possible variations it may be assumed, on the basis of observations of the irritability of nerves *in situ*, that those in which the action-potential does not become negative for the second time, most closely resemble what happens when the nerve is in its normal physiological state.

The variations are important because of the light that they shed upon the composition of the action-potential. The first positive wave is the homologue of the positive wave as it regularly appears in a single response. A tetanus accentuates it. At the same time it causes an increased production of negative potential. According to the size and duration of the latter, the position of the positive trough is determined and also the amount of negativity residual when the opposition of the first positive wave is removed. Otherwise stated, the first positive potential is the positive potential of the single action—augmented—and intercurrent in a negative potential also augmented by the tetanus. The second positive potential must be connected with the tetanus through the increased negative potential. An increased negative potential is regularly followed by an increased positive potential and must be complemented by it.

The effect of an increased negative after-potential in a condition in which there is no simultaneous increase of the first positive potential is seen in single responses of veratrinized nerves (figs. 3 and 7). As the negative after-potential develops, it fills in the positive trough on the early side and creates new positivity beyond it; and this process goes on until the negativity lasts 30 sec. and the succeeding positivity 100 sec. The latter figures are taken from experiments with the string galvanometer in which the size of the negative after-potential attained was 1.6 mv.

Comparison of the potentials after a tetanus with the augmented potentials following a single response of veratrinized nerve reveals an analogy and a contrast. In both there is an enlarged negative after-potential followed by a positive potential; but after a tetanus there is an additional variable—an augmented positive component cutting into the negative after-potential.

When a veratrinized nerve is tetanized, the negative after-potential is so dominant that all signs of an augmented first positive potential are obliterated. Staircasing of the negative after-potential occurs to a marked degree during the tetanus; at the end of the tetanus the after-potential starts with the high negative value which has been reached and declines to the analogue of the second positive period, without sign of a first one (fig. 8).

EXCITABILITY IN RELATION TO THE ACTION-POTENTIAL. The measurement of excitability means the determination of thresholds from the begin-

ning of the action to the end of the last trace of potential change. Negative after-potential is associated with supernormal excitability (Gasser and Erlanger, 1930), positive after-potential with subnormal excitability (Graham and Gasser, 1934; Gasser, 1935). Therefore, if the rule be generally applicable, the curve of excitability should be as characteristic of the nerve and its various states as is the action-potential. We shall see that the rule holds.

Refractory period. The end of absolute refractoriness should come with the ending of the spike (Adrian, 1921), that is, very soon after 0.4 msec. In the best preparations this duration is indeed approached; refractory periods lasting between 0.41 and 0.44 msec. have been obtained in both roots and peripheral nerve. But not infrequently, no sign of a second response can be evoked unless the shock interval is 0.5 msec. The earliest responses are small and slow of conduction, but even a slight lengthening

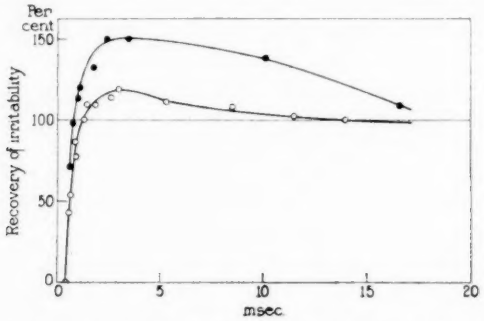


Fig. 9. Recovery of excitability in an isolated saphenous nerve (circles) and a phrenic nerve (dots).

of the interval brings out a much larger size. At the same time, the threshold falls, and at intervals as small as 1.0 msec. supernormality may already be reached (fig. 9). We shall see later that recovery of isolated nerves takes place more rapidly than recovery of nerves in the body.

From the shortness of the refractory period the inference may be drawn that a nerve can produce 2000 spikes or more per second. Upon testing the inference it is found that a rate of this kind is possible, but for short periods only, and when the stimuli are decidedly supermaximal and the lead is close to the stimulating cathode. Figure 10 shows 2000 spikes per second (upper line), combined with shock artifacts of the magnitude shown in the lower line. Some fibers are alternating, but the spike areas produced cannot be accounted for otherwise than by assuming that some of the fibers are responding to every stimulus. Records made at a distance from the cathode, when the frequency of stimulation is 2000 per second are not susceptible of a detailed interpretation, because of the confusion of tem-

porally dispersed spikes and shock artifacts. One point, however, is clear. The amount of alternation occurring is much greater at a distance than near the cathode, showing that many of the impulses set up by the strong shocks encounter some point in the nerve which they cannot pass.

At 1 msec. recovery has so far advanced that a second response has a large fraction of normal size, the fraction depending upon the refractory periods of all the fibers of the nerve and upon the state of the nerve. For example, even the threshold fibers of the two nerves presented in figure 9 would not produce maximal spikes at the same intervals. None of our preparations has ever shown complete recovery of size in 1 msec. Eighty per cent recovery, as seen in figure 11, is a fair example. Spikes cannot be maintained at this level, however, when they follow one another at millisecond intervals. Even in the first line of figure 11 some falling off is apparent, and the decrease progresses continuously until some fibers begin to drop out and alternation sets in (third line).

Supernormal period. Following the relatively refractory period, the excitability of isolated nerves becomes supernormal. In many preparations the excitability rises to between 120 and 150 per cent of normal (fig. 9), occasionally to 200 per cent, and the supernormality lasts as long as the negative after-potential, i.e., 12 to 20 msec.

A striking method of demonstrating the supernormality during the negative after-potential is to interpolate a maximal response in the course of a series of stimuli at threshold. A description of the method follows. Through one pair of electrodes shocks are applied to the nerve from a thyratron stimulator at a rate of more than 100 per second, so that the individual shocks will not be spaced by an interval longer than the supernormal period. The strength of stimulus selected is just above threshold; a few fibers are stimulated, but for most of the fibers of the nerve the strength is subminimal. Through a second pair of electrodes, placed at a greater distance from the leads, a single maximal shock is applied. When the impulses initiated by a single shock are conducted past the pair of electrodes, through which the shocks from the thyratron are being applied, one of the latter will fall within the period of supernormality of the action and then, instead of being able to produce a response only in threshold fibers, will be able to stimulate all the fibers of the nerve. The next thyratron shock in turn will also fall in a supernormal phase, and thus the cycle is repeated (fig. 12).

A train of maximal responses cannot be set up, however, without further consequences. At the beginning of the train the negative after-potential increases, giving the appearance of staircasing in both the spikes and after-potentials (Gasser, 1935). Later, along with the tendency to produce positive after-potential, the threshold rises and fibers begin to drop out in the order of their thresholds. Once a fiber has dropped out it cannot

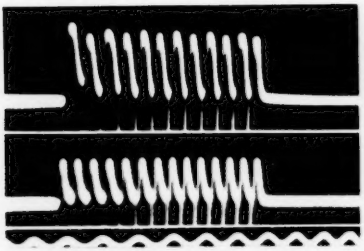


Fig. 10

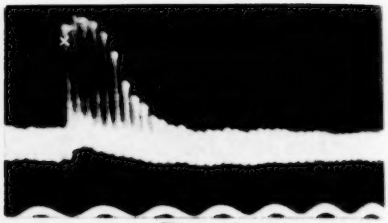


Fig. 12

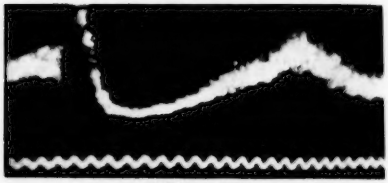


Fig. 13

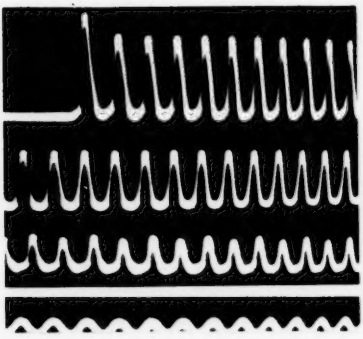


Fig. 11

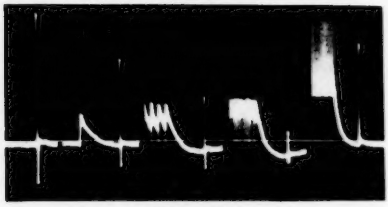


Fig. 14

Fig. 10. Upper line: Short tetanus of a dorsal root at 2000 per second. Lower line: Shock artifacts recorded during reversible ether narcosis (at a slightly higher frequency). Time: 1 msec.

Fig. 11. Start and progression of a tetanus in a phrenic nerve. Frequency of stimulations: 1000 per sec. The third line follows the first by about 20 sec. Time: 1 msec.

Fig. 12. Effect on a threshold tetanus of an interpolated maximal shock, phrenic nerve. Tetanus at 325 per sec. Interpolated shock at *x*. Time: 20 msec.

Fig. 13. Effect of the negative and positive phases of the after-potential in a phrenic nerve upon spontaneous discharge. A single conditioning action starts at the break in the line. Time: 10 msec.

Fig. 14. Excitability in relation to the depth of the positive after-potential in the phrenic nerve. Varying depths of positive after-potential are produced by tetani of different lengths and frequencies. Testing shock applied through another electrode and at the same strength in each case. a, testing response in isolation; b, testing response after single conditioning action; c, d, e, after tetani. The added horizontal line is at zero potential. Vertical projections below the line are diphasic artifacts.

reënter, because at the time of arrival of the next shock, the irritability will have changed to subnormal. Thus the response of the nerve as a whole to the thyratron stimuli gradually returns to the condition which obtained before the interpolated shock occurred.

Subnormal period. After the supernormal phase of a single response there develops a period of subnormality, which ordinarily lasts as long as any degree of difference from the normal threshold can be detected. In the case of rhythmic nerves, however, the situation is different. While the entire course of the excitability in these nerves has not been plotted by the method of thresholds, enough points have been located to prove that the supernormality reappears with the start of the second negative potential, and disappears again with its subsidence. Successive conditioning actions, when rhythmic in character, show enough minor differences to make the application of the tedious method of plotting recovery by thresholds of doubtful value; however, a qualitative indication of the course of the excitability can be derived from the behavior of nerves discharging spontaneously. The killed end (Adrian, 1930) serves as a continuous stimulus, and the amount of the discharge follows the irritability of the nerve. During the first negative after-potential the spontaneous discharge is greatly augmented. As the negativity declines and the potential enters into the first positive portion, the discharge decreases, and it may be extinguished when the positivity reaches maximum (fig. 13). As the potential rises to a second negative maximum, the discharge increases again, and at the crest it is once more greater than in the undisturbed, steady state. The same cycle is repeated in an attenuated form in the approach from the second maximum to the third and fourth.

During the prolonged and augmented potentials following a tetanus, the relation of potential to excitability still holds; but for want of sufficient data no precise statement can be made as to how the excitability changes with the potential. That it does change may be shown by determining the subnormality at the maximum of the first positive trough. As the latter deepens at the end of tetani of increasing lengths or frequencies, the subnormality also increases (fig. 14, a, b, c, d). At the end of tetani of such extent that the absolute level of the maximum begins to rise again, because of the magnitude of the negative after-potential in which the first positive potential is an incisure, the subnormality begins to decrease (fig. 14 e). At the end of the incisure, subnormality gives way to marked supernormality.

In order to test the excitability during the remainder of the after-potential following a tetanus, a very simple procedure was followed. The amplifier was set to give about 30 mm. per mv. and a shock, thyratron-controlled as to strength, was selected to give a spike of about one-fifth of the maximal when the nerve was equilibrated to one response per two seconds. Such spikes were good indicators of changes in irritability. If

the threshold fell, more fibers were stimulated and the spikes became higher; if the threshold rose, fewer fibers were stimulated and the spikes became lower. In a series of equilibrated responses a short maximal tetanus (starting from electrodes placed on another portion of the nerve) was interpolated, and the sizes of the responses were photographed during successive sweeps of the oscillograph before and after the tetanus.

The results obtained in a sample experiment are plotted in figure 15. A very striking increase in the size of the responses occurs during the negative after-potential, and this is followed by a decrease during the positive after-potential. Thus the complete cycle through which the excitability of a fiber in such a nerve passes before returning to its steady state is made up of: the refractory period, a first supernormal period, a first subnormal period, a second supernormal period, and a second subnormal period.

In the form just described, the relation of excitability to the after-potentials is clearly defined: supernormality and subnormality in relation to negative and positive potentials; but the form is an exaggerated one, resulting from the condition of nerves in isolation. The form depends upon

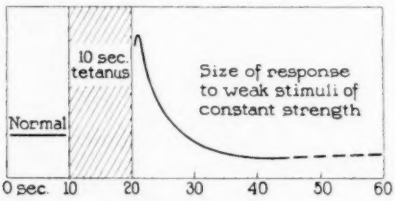


Fig. 15

both the degree of previous activity and the length of time the nerve has been mounted. Fresh nerves show less negativity and less supernormality.

Through the exaggerated form, the way is opened to an understanding of the normal form. The same processes are operative when a nerve is functioning in its proper position in the body and their presence is recognizable by the characteristic shape which they impart to excitability curves. Although the processes operate to a different extent and their effects sum up to give a quantitatively different picture, the individual features of the curve are identifiable with similar curves seen in isolated nerve. For example, if the recovery of excitability, instead of following a smooth curve, shows an early acceleration of recovery which is not maintained and is succeeded by a recession, it may be inferred, even if no actual supernormality occur, that a process making for supernormality is playing its part.

Excitability of nerve in situ. In order to gain information about the extent to which the various potentialities of a nerve are exercised when it is functioning as it does physiologically, an examination was made of nerves

in the body. After-potentials cannot be measured on a nerve *in situ* without exposing the nerve to an extent which precludes all assurance of its being in a physiological state. On the other hand, thresholds may be measured without exposure of the nerve; and through the associations existing between thresholds and potential, the nature of the latter may be inferred.

Thresholds were measured on the saphenous nerve of the cat in intact animals under general anesthesia, or in decerebrate or chronic spinal preparations. An incision was made through the skin in the upper part of the thigh over the saphenous nerve, without making a further dissection. The nerve was, therefore, unexposed and left with its natural circulation intact. Two pairs of chloride-covered silver-wire stimulating electrodes were placed in a vertical position over the surface of the thigh, in a way so that their tips came in contact with the fascia immediately overlying the nerve. The conditioning stimuli were supplied through the proximal electrodes, and the test shocks through the distal electrodes. In this way, local effects were avoided. Precaution was taken to prevent cooling of the limb. In many preparations the nerve was blocked intra-abdominally by a ligature, in order to prevent interference by reflex movements.

The effect of testing shocks was recorded from a portion of the nerve exposed below the knee. The nerve was drawn through a glass tube, into the sides of which silver wires had been sealed in order to make connection with the amplifier and oscillograph. When the tube was fastened in place, it served as a very effective moist chamber, the nerve staying in good condition for hours. It was, of course, not essential to the experiment that this part of the nerve be in a completely physiological condition, as it served merely as an indicator of whether or not a response had been started by the testing stimulus applied to the protected central portion of the nerve. Throughout all the measurements the testing shocks were adjusted to a strength which would produce a response of constant height, the amplification being such that the height selected involved a small group of the lowest threshold fibers of the nerve. Excitability was thereby defined as the reciprocal of the shock strength which would excite a constant number of fibers. As variation in the recovery curves would be expected chiefly in the portions connected with supernormality and subnormality, the course of the recovery was not plotted earlier than the later part of the relatively refractory period. Thereby the nerve was spared exposure to the strong shocks necessary for measuring early recovery.

A few early experiments were performed under general anesthesia with ether or dial, but in order to avoid possible effects of the narcotics, most of the experiments were carried out on decerebrate preparations, from which the ether administered during the decerebration had been completely eliminated. The nerve was then found to remain for six hours or longer in a steady state suitable for experimentation.

As the activity of nerve is known to be susceptible to changes in the acidity of the medium about it, and because of the possibility of the production of an acidosis in the process of narcotization and decerebration, a short, final series was carried out on chronic spinal cats. Samples of blood were taken from the latter for determination of the pH; the values found ranged between pH 7.3 and pH 7.4.[1] As between the two reactions, no significant difference could be made out in the excitability curves. Whatever change was entailed by a reaction of pH 7.3, in place of the normal pH 7.4, was obliterated by the random variations. Nor was there any significant difference between this series and the one carried out on decerebrate animals; consequently we believe that the curves as described fall within the limits of what may properly be called normal nerve functioning.

In the body, as outside, the excitability curve is characterized by a rapid rise to a maximum, a decrease from the maximum, and a long subnormal period. In nearly all the experiments, in which the conditioning excitation was a single shock, the maximum was at a definitely supernormal level (in 27 of 30 decerebrate preparations, in 4 of 5 with dial, in 2 of 3 with ether, and in all the chronic spinal preparations). Relative refractoriness ended and supernormality began 2.5 to 5.0 msec. after the start of the action. Maximum supernormality was reached after 5 to 10 msec., and the transition from supernormality to subnormality occurred at 12 to 18 msec. Subnormality was greatest at 25 to 35 msec., and there was a return to normal at 60 to 80 msec. (These durations are the ones which guided the designation in an earlier section of the paper of what must be the normal duration of the after-potentials.) The absolute degrees of supernormality and subnormality varied somewhat from animal to animal. Supernormality varied from 0 to 20 per cent at the peak, with an average of 7 per cent. Subnormality varied from a just detectable amount to 6 per cent, with an average of 3 per cent.

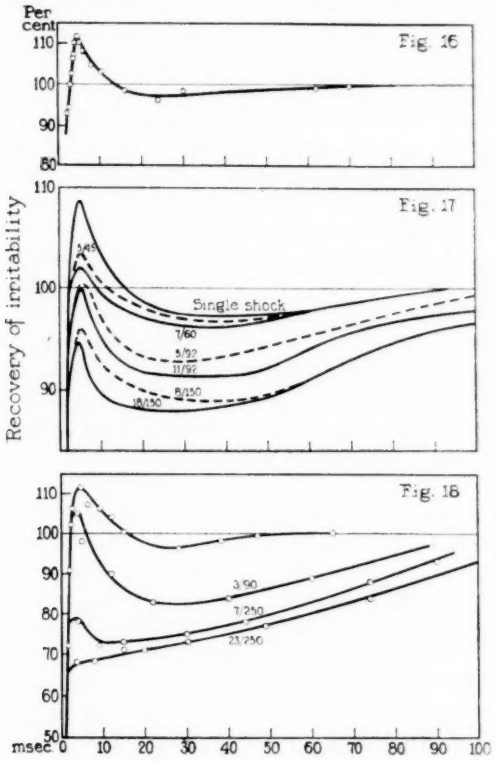
Figure 16, taken from one of the chronic spinal preparations, shows a typical curve. Compared with the curves obtained from isolated nerves, the principal difference is found in the supernormal phase, which is less in degree and shorter in duration. The relatively refractory period is longer. In isolated nerve the relatively refractory period is shortened by the augmented negative after-potential process, in accordance with a mechanism analogous to that described by H. T. Graham (1934) for frog nerve.

When the number of shocks in the conditioning excitation was increased, the same general form of excitability curve was found, but at all intervals between the conditioning excitation and the testing shock, the excitability

[1] We are indebted to Dr. J. E. Lehmann for the making of the measurements and to Dr. L. Michaelis for the use of his glass electrode and accessory apparatus.

was decreased, and in relation to the degree of decrease the period of subnormality was extended.

Figure 17 presents a family of curves in which a comparison is made on the same nerve of the course of recovery following a single conditioning shock, with that resulting from conditioning tetani of various duration and



Figs. 16, 17, 18. Recovery of the saphenous nerve of the cat *in situ*. Figure 16, chronic spinal cat. Single conditioning shock. Figures 17 and 18, observations on two nerves in decerebrate animals. Conditioning action changed in the course of the experiment from a single shock to tetani. The number of shocks used and their frequency are indicated; e.g., 5/45 means 5 shocks at 45 per sec. Figure 18, upper curve conditioned by a single shock.

frequency. A few shocks in the conditioning tetanus were sufficient to bring the initial maximum below the level of 100 per cent excitability, and a greater number carried it still lower. High frequency of shocks was more effective in this respect than a large number of shocks. At the same time, the decline in excitability following the maximum was more rapid, the

maximum of subnormality came earlier, and the area subtended by the initial peak rise was decreased. When a still more intense conditioning stimulation was applied, the peak disappeared altogether, all that was left being a shoulder on the curve of recovery. Figure 18, taken from another nerve, shows the course during a development of this kind. Here a subnormality amounting to 30 per cent was produced. The after-potential which would correspond to the lower curves in this figure would in all probability resemble that pictured in figure 6 c.

Long-lasting subnormality follows severe tetanization. In one experiment a tetanus for 15 sec., at the rate of 300 per sec. produced subnormality for 20 sec., and a similar 30 sec. tetanus, subnormality lasting 30 sec. In another experiment, subnormality of one minute succeeded a tetanus lasting one minute.

In the description of the experiments on isolated nerve the prolongation of the positive after-potential by tetanization was pointed out. Despite the aid to be derived from an active circulation, a similar process must occur in the body; otherwise the subnormality could not be accounted for. The amount of negative after-potential, on the other hand, must be very much less than in isolated nerve. Augmentation of the latter to the point of introducing an intercalated supernormal phase, as happens in isolated nerve, must be considered as being a distinctly unphysiological distortion. A search for a second period of supernormality in the body has thus far led to negative results only; but whether or not there are any signs of an analogous effect can only be decided after the mapping of recovery following a severe tetanization has been given a more careful study than any heretofore attempted.

Discussion. One of the most important questions in connection with this investigation has been: Is the supernormal phase physiological? On the basis of our experience, we feel that it is physiological. While our experiments were in progress, observations made on the recovery curves of rabbit oculomotor and sciatic nerves *in situ* were published by Lorente de Nó and H. T. Graham (1935). Our findings are completely in agreement with theirs. They found, however, that in rapidly made preparations, in which readings could be started within an hour after the beginning of the experiment, supernormality was absent, although it appeared later; and they raised the question whether the true normal state is not better represented in these early observations than in the later ones.

We have not examined nerves during the first hour after decerebration. Our observations were all started later than one hour, and they extended over a period of many hours. During this long period the nerve remained in a state which was satisfactorily constant, and we had every reason to believe that this period—rather than an early one, in which possible effects of residual anesthetic or abnormal breathing arising from the manipulation

might have operated—offered the most favorable time for the making of readings. Confirmation of the results so obtained by those derived from chronic spinal cats supported that opinion. Also, no difference was found between curves obtained soon after application of the electrodes and those obtained hours later. Consequently we feel that the nerves used were well within the limits in which nerves must function during ordinary body activity.

While the existence in the body of a process making for supernormality may be considered as being established, absolute supernormality cannot be of great importance to a nerve in its function of carrying messages, as it is seen only when the excitability after a single response, or a very few responses, is compared with that of a quiescent nerve; that is, under conditions which differ from the ordinary mode of occupation of nerve fibers by actions. Nerve messages are carried by a series of actions, and following a series absolute supernormality is not seen in nerves *in situ*. The importance resides rather in the process which produces supernormality. *The recovery of excitability must be greatly hastened by it.* Instead of following an ordinary exponential curve, the course of recovery shoots far ahead of a curve of this kind and reaches an early maximum, although it does not achieve absolute supernormality. In the exaggerated form in isolated nerve, the acceleration of recovery is such that excitability may rise from absolute refractoriness lasting more than 0.5 msec. to supernormality occurring earlier than 1.0 msec.

From studies made of peripheral nerve it is desirable to get a composite picture of the properties of nervous tissue; therefore, a comparison of mammalian and frog nerves is useful. The idea that there is a set of common properties is supported by the fact that all the phenomena which have been worked out on frog nerve may be recognized qualitatively in mammalian nerve: a negative after-potential varying with the conditions about a fixed spike; a relatively refractory period shortened by development of the negative after-potential process; supernormality varying with the amount and duration of negative after-potential; a positive after-potential augmented by tetanization or by conditions which increase the negative after-potential; and subnormality associated with the positivity.

The points which are shown more clearly by mammalian nerve than by frog nerve are: positivity after a single response; the division of the positive after-potential into two parts; and the concurrence of processes which, on the one hand, tend to produce negativity, and on the other hand, positivity.

On the theoretical side the impression has gained support that the first positive potential is associated with spike production, and that the second positive potential is secondary to the negative after-potential process. A simple sequence of negative and positive after-potentials is seen in single responses of veratrinized nerves. The same sequence at the end of a

tetanus of unpoisoned nerve is complicated by the augmented first positive potential cutting into the negativity.

SUMMARY

The properties of the action-potential of mammalian A fibers are described.

Measurements of the duration of the spike in single fibers of spinal roots show that the major portion of the spike is completed in 0.4 to 0.43 msec.

The spike is succeeded by processes giving negative and positive potential signs. These potentials sum algebraically and the absolute value of the potential at any time depends upon the individual momentary values of the potentials. Immediately following the spike, the after-potential is negative. The negative after-potential varies independently of the spike, and in veratrinized nerve it may be shown to have a rising phase. Succeeding the negative period, a positive after-potential is always present in normal fibers. On the average its magnitude amounts to 0.2 per cent of the spike height.

In freshly mounted isolated nerves, in which the fibers are subject to spontaneous discharges, the after-potential is rhythmic.

Tetanization increases both the negative and the positive after-potentials. The positive potential then appears in two parts. The first part is analogous to that following a single spike; it has the same duration as the latter, but is larger. The second part has a duration as well as a size which is related to the severity of the tetanus. After a maximal tetanus of 30 sec. the potential is + 0.6 to 0.7 mv., and the duration more than four minutes. The increased negative after-potential may cause the two parts of the positive potential to be separated by a period of absolute negativity, or in other instances by a negative crest still on the positive side of zero. The variations are described.

The augmented negative after-potential of veratrinized nerve is succeeded by a large positive potential which seems to be analogous to the second part of the positive potential following a tetanus.

The refractory period lasts 0.41 to 0.44 msec. in the best preparations. Tetani at 1000 and 2000 per second are described.

Recovery of excitability was studied in nerves functioning under physiological conditions in decerebrate and chronic spinal cats. A typical set of excitabilities in a single response may be described as follows: relatively refractory period, 3 msec.; supernormality between 3 and 15 msec., with a maximum of 7 per cent at 7 msec.; subnormality between 15 and 70 msec., with a maximum of 3 per cent at 30 msec. The durations of the supernormality and the subnormality define the normal durations of the negativity and positivity of the after-potential.

Following a tetanus, the excitability as shown in all parts of the curve is

depressed, but except after very severe tetani, the typical form of the curve is preserved: a rapid rise, a secondary fall, and a slow recovery. The total duration is increased in relation to the severity of the tetanus. A one minute tetanus is followed by subnormality of one minute.

In isolated nerves in oxygen there are quantitative differences from the condition obtaining in the body. The relatively refractory period is shorter and the supernormality greater, because of an abnormally large negative after-potential. Following a tetanus, the excitability depends upon the course of the after-potentials. In cases where the negative after-potential is sufficiently augmented along with the positive after-potential, the course is: relatively refractory period, first supernormal period, first subnormal period, second supernormal period, and second subnormal period.

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THE UTILIZATION OF FRUCTOSE IN THE MAMMALIAN ORGANISM AS SHOWN BY EXPERIMENTS ON HEPATECTOMIZED AND EVISCERATED PREPARATIONS[1]

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The preponderant weight of opinion has been in favor of the view that carbohydrate foods are first converted to glucose before oxidation in mammals, and much attention has been focussed therefore upon the mechanism and site of such conversion. The extent to which other sugars can substitute glucose has been regarded as depending upon the rate at which they were converted to glucose. On the other hand, it is still a moot point as to whether sugars other than glucose are always converted to glucose before they can be oxidized by the different tissues of the mammalian organism. The experiments recorded in this paper were undertaken in the hope of elucidating these problems in the instance of the sugar fructose, a common constituent of our diet, chiefly in the form of cane sugar.

Carbohydrate foodstuffs are absorbed into the portal system from the intestine in the form of monosaccharides. What chemical changes, if any, take place during passage through the wall of the intestine have not been definitely established. While it has been conclusively shown that fructose can be absorbed as such, that a certain amount of conversion to glucose may and does take place during absorption is a fact not yet demonstrated.

That the liver possesses the ability to convert fructose to glucose has been conclusively shown by a number of workers in *in vitro* and *in vivo* experiments. Indeed this well-known ability of the organ to effect this conversion has been used extensively as an efficiency test of hepatic function under varying conditions. But in passing it should be mentioned that the work of Mann and Bollman (1926) demonstrates the extreme unreliability of this test.

Bollman and Mann (1931) showed that the liverless dog could be maintained in as good physiological condition for a considerable period by an infusion of fructose as by that of glucose. These authors also found an increase in blood glucose when fructose was injected intravenously into the

[1] A report of this work was given at the Annual Meeting of the Royal Society of Canada, May, 1935.

liverless dog. This finding they have interpreted as indicating that fructose is first converted into glucose, and the glucose so formed then oxidized in these animals. Further, the fact that they were unable to show any utilization of frucose in their eviscerated dogs suggested to them that the mucosal cells of the intestine are responsible for such conversion in the liverless animal.

EXPERIMENTAL PROCEDURES: *Chemical.* True blood sugar values of our animals were obtained on Somogyi zinc hydroxide filtrates using the modified copper reagent of Shaffer and Somogyi (1933). In the absence of fructose the figures indicated the true glucose content of the blood. For the estimation of fructose we have employed two methods, that of Harding and Nicholson (1933), and the method of Campbell and Hanna (1926). The former is a fermentative method in which use is made of the selective action of a certain strain of *proteus vulgaris*. This organism quantitatively removes glucose from dilute solution, while fructose is unattacked when present in low concentration. The stock culture[2] of this organism has been maintained in nutrient broth and when required planted out on glycerine-agar. These departures from the method as originally given lead to considerable simplification of this part of the work and in no way diminish the yield of growth nor its fermentative properties. Given adequate facilities for the culturing in bulk of the *proteus vulgaris* we regard the method as eminently feasible. Even so, whenever this method was employed, we have always conducted control tests on the fermentative powers of the carefully washed organisms, their ability to remove completely from solution an amount of glucose greater than that of any of the blood filtrates under test, and further their inability to remove any fructose. In the original method tungstic acid filtrates of blood were used. We have employed this organism to remove glucose from zinc hydroxide filtrates. The small amount of zinc which remains in the filtrate does not affect the glucose-removal power of the organism. After centrifugation of the filtrates following incubation with the *proteus vulgaris*, the opalescent supernatant can be completely cleared by shaking with a little Lloyd's reagent and filtering through fluted filter paper.

A chart for use with the Campbell and Hanna permanganate titration method of fructose estimation was constructed based on recovery figures of fructose when added to tungstic acid blood filtrates.

Operative. The hepatectomy operations were carried out on dogs

[2] We are greatly indebted to Doctor Nicholson, of the Department of Pathological Chemistry of this University, for supplying us with the initial strain of *proteus vulgaris*, and for help during our preliminary trials of the method. Our thanks are also due to Dr. D. T. Fraser, Department of Hygiene and Preventive Medicine, for helpful suggestions relating to the growth, and for generous supplies of cultures of this organism throughout the course of the work.

according to the one-stage technic described by Markowitz, Yater and Burrows (1933). The method of evisceration employed was suggested to us by our colleague Dr. J. Markowitz.

The essential departure from the customary technic is the use of a pyrex glass cannula in the inferior vena cava, as in the hepatectomy operations, which is a distinct improvement over the usual practice of ligating this vein and trusting to the establishment of an adequate collateral circulation. The urinary bladder, kidneys and suprarenal glands were not removed. These animals made good recoveries.

It was found that clamping the lower end of the esophagus resulted in persistent retching movements following recovery from the anesthetic,

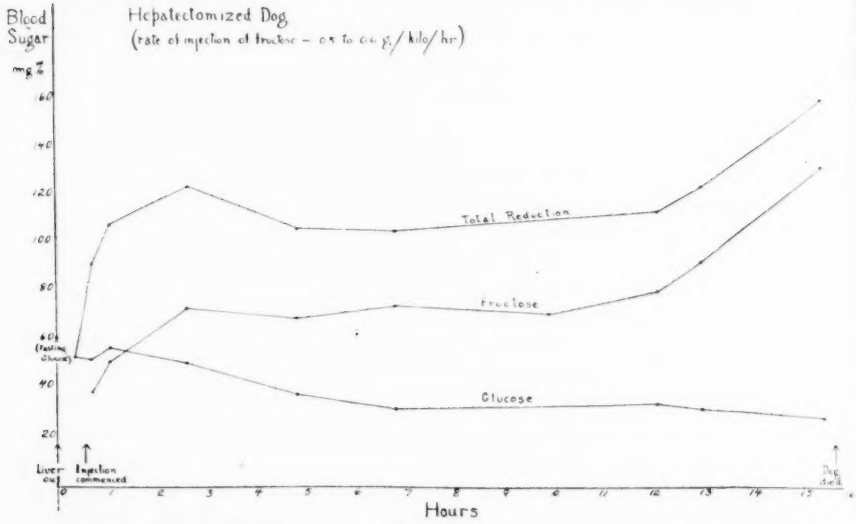

Fig. 1. ♀ Weight = 11.0 kgm. Pre-operative fast 24 hours. P.M. findings: negative. Total bloody fluid in abdomen 75 cc. (cell vol. = 11 per cent).

whereas when the clamp was placed one centimeter below on the walls of the cardiac end of the stomach these movements were considerably diminished. The troublesome ropy mucus was removed from the esophagus by washing out with several lots of water.

EXPERIMENTAL RESULTS. For the sake of brevity single experiments only are reported, but the results of these experiments have been amply confirmed in several other experiments conducted on similar preparations. The glucose-free fructose was usually administered in a 25 per cent solution containing 0.8 per cent sodium chloride, by continuous intravenous injection into the saphenous vein, using a constant injection pump.

Figure 1 shows the blood sugar levels in a hepatectomized preparation

TABLE 1

Concentrations of glucose and fructose in the blood of hepatectomized dog after intravenous injections of fructose

SAMPLES	TIME AFTER REMOVAL OF LIVER	1	2	3	4	5	6	7	8
		METHOD							
		Zinc precipitation and proteus fermentation				Tungstate precipitation and $KMnO_4$ titration			
		Total reduction	Fructose		Glucose 1-2	Total reduction	Fructose		Glucose + non-fermentable reduction 5-6
		Calculated as mgm. per cent glucose	Calculated as mgm. per cent glucose	Mgm. per cent fructose	Mgm. per cent glucose	Calculated as mgm. per cent glucose	Calculated as mgm. per cent glucose	Mgm. per cent of fructose	Calculated as mgm. per cent glucose
1	Pre-operative	72 3			72 3	86 0			86 0
2	30 min.	81 7			81 7	99 7			99 7
3	46 min.	67 7			67 7	80 5			80 5
0.5 gm. fructose per kgm. injected	46 min. to 48 min.								
4	57 min.	198 0	126 0	134 0	72 0	210 0	129 8	138 0	80 2
5	1 hr. 25 min.	136 7	72 7	76 7	64 0	152 0	81 7	86 9	70 3
0.5 gm. fructose per kgm. injected	1 hr. 25 min. to 1 hr. 26 min.								
6	1 hr. 37 min.	218 0	156 7	166 3	61 3	238 0	166 3	177 0	71 7
7	2 hr. 5 min.	151 3	94 3	100 0	57 0	168 7	100 5	106 9	68 2

The fructose concentrations as estimated by the $KMnO_4$ titration (column 7) are higher than those obtained after proteus fermentation (column 3). The reason for this discrepancy is not clear although it may be accounted for in part by the rather indefinite end-point of the $KMnO_4$ titration. In other experiments the agreement was frequently much closer. However, by neither method was any rise in blood glucose indicated after the ingestion of fructose. It should be stressed that the difference in reduction of the copper reagent by glucose and fructose is taken into account and is shown in columns 2 and 3 and 6 and 7. To obtain the correct glucose concentration the fructose must be calculated as glucose and subtracted from the total reduction which is also calculated as glucose. Although the $KMnO_4$ method estimates fructose directly, each figure so obtained was converted into the equivalent glucose figure as would be given by the copper reduction method. This value could then legitimately be subtracted from the total reduction (as mgm. per cent glucose) of the blood sample to give the glucose content plus the non-fermentable reduction in the tungstate filtrate. There is no significant variation in the non-fermentable reduction values of such blood filtrates. The table will suffice to show that inattention to this difference in the copper reducing powers of glucose and fructose would lead to very appreciable errors especially in those samples containing relatively high concentrations of fructose.

receiving a continuous intravenous injection of fructose at constant rate. The concentration of fructose in the blood remains practically the same until towards the end of the experiment, when there is an increase in the fructose concentration showing a marked decrease in utilization of this sugar prior to the death of the animal. It will be seen that the concentration of blood glucose remains remarkably constant after the initial fall.

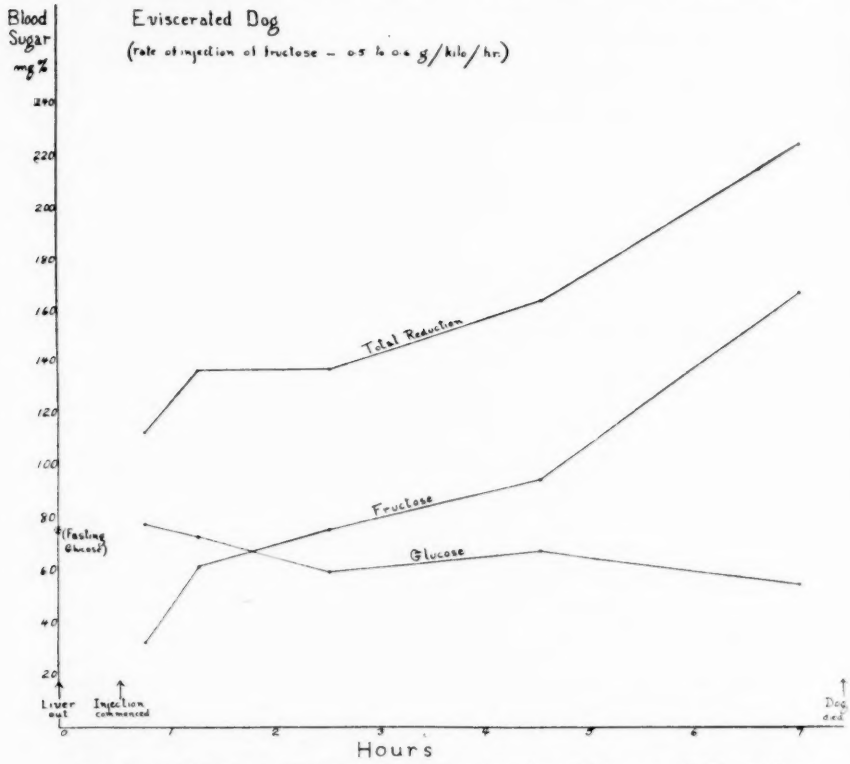

Fig. 2. ♂ Weight = 9.3 kgm. Pre-operative fast 24 hours. P.M. findings: negative.

One interpretation of this constancy of glucose concentration would be that the fructose was converted to glucose at the same rate as that of the oxidation of glucose. But the fact that no change occurred in the concentration of blood glucose on varying the rate of injection of fructose leads us to doubt the validity of this hypothesis. Thus in table 1 it will be seen that there is no appreciable change in the concentration of blood glucose, even when the fructose is injected rapidly in rather massive amounts. In

this respect our results are in definite contradiction to those of Bollman and Mann, who found a marked increase in the concentration of blood glucose in the hepatectomized animal following such an injection of fructose. These authors used the Campbell and Hanna method for estimating blood fructose. We are unable to offer any explanation for this divergence of experimental results. We have repeated this experiment several times, both methods of fructose estimation have been used, and in no case have we obtained either an increase or a decrease in glucose concentration which was outside the limits of our experimental error. We regard this experimental finding as of considerable importance in elucidating the problem as to whether conversion of fructose to glucose necessarily takes place prior to oxidation. Bollman and Mann could find no other obvious interpretation for their experimental result, and indeed it was almost entirely upon this finding that they favored the conversion theory.

Figure 2 shows the levels of the blood sugars in an eviscerated preparation receiving a continuous intravenous injection of fructose. While the glucose concentration shows little variation, the decrease in rate of utilization of fructose towards the end of the experiment is again apparent. Neither this animal nor similarly treated animals showed any sign of hypoglycemic convulsions. They lived for about $6\frac{1}{2}$ to $7\frac{1}{2}$ hours after completion of the operation. The longest survival period of control eviscerated dogs receiving no sugar or other injection was three hours. In some of these experiments, a determination of free sugar of muscle was carried out seven hours after the evisceration. No attempt was made to differentiate between glucose and fructose, but the total free sugar was in accord with expectation and at least serves to show that there was no undue accumulation of free sugar in muscle, and hence further assurance that the injected fructose was definitely being utilized by the eviscerated animal. In our eviscerated preparations the inferior vena caval blood flow was uninterrupted, whereas in Mann and Bollman's experiments the inferior vena cava was tied and the establishment of an immediate efficient collateral circulation relied upon. The difference in operative procedures may account at least in part for the evident difference in behavior of their preparations and ours following the administration of fructose.

One of the hepatectomized preparations following the belated administration of fructose, given continuously at the rate of 0.4 gram per kgm. of body weight per hour, began to show slight convulsive movements about two hours after the commencement of injection. The blood glucose concentration at the time of the onset of muscular twitchings was 25.9 mgm. per cent; that of fructose 55.7 mgm. per cent. In spite of the marked increase in the rate of fructose infusion to 1.3 gram per kgm. per hour there was no material increase in the glucose concentration (27.0 mgm. per cent after $\frac{1}{2}$ hour, when fructose value was 104 mgm. per cent) and hypoglycemic

symptoms persisted. But there was no progressive increase in the severity of the movements, the symptoms remaining of a very mild type over a period of three-quarters of an hour. An injection of glucose ($\frac{1}{2}$ gram per kgm.) at this stage was promptly followed by complete disappearance of all hypoglycemic symptoms and well-marked general improvement in the condition of the animal. Although the hypoglycemia was not abolished by the fructose we feel that this sugar prevented the development of more vigorous symptoms.

Discussion. In the interpretation of our experimental results we feel due consideration must be given to the fact that in both hepatectomized and eviscerated preparations receiving fructose there is no material increase in the blood glucose concentration. We should like to stress that as far as a review of the experimental data is concerned, there is nothing at variance with the hypothesis that in mammals fructose can be directly oxidized. While in some types of tissues fructose may be directly oxidized, in others this may not be possible, conversion to glucose being first necessary. This conversion may take place in such tissues or the glucose may be derived from a possible conversion of fructose to glucose by some other tissue in excess of its own carbohydrate requirements, the net result being no substantial alteration in the total quantity of free glucose as indicated by the constancy of the blood glucose concentration.

The two chief tissues concerned with the consumption of carbohydrate are the skeletal musculature and the brain. Bornstein and Völker (1929) showed that a perfused isolated mammalian limb utilized 0.25 gram fructose per kilogram as compared with 0.225 gram glucose. The muscles may first convert the fructose to glucose which is then oxidized, or the oxidation of fructose may take place directly. Regarding the energy requirements of brain, Holmes (1934) has recently stated "There is now abundant evidence that the brain as a whole has a high metabolic rate, and that its metabolic demands are chiefly for carbohydrate." Loebel (1925) first showed that brain tissue can utilize fructose without preliminary conversion to glucose, for no lactic acid appears to be formed as is the case with the metabolism of glucose in brain tissue.

Bollman and Mann were unsuccessful in their attempts to alleviate the symptoms of extreme hypoglycemia in the liverless animal by administration of fructose. We failed to abolish mild hypoglycemic convulsions with fructose, but we feel the development of more vigorous symptoms was prevented. Administration of fructose did not lead to any increase in the blood glucose, nor presumably in the glucose concentration of the tissues, and the symptoms persisted. But the *in vitro* experiments of Loebel with brain and of Gerard and Meyerhof (1927) with nerve trunks and later those of other workers show such tissues can oxidize fructose as readily as glucose. The cause of the nervous stimulation of hypoglycemia therefore

appears to be due specifically to a marked lowered glucose concentration or that of a glucose intermediary (cf. tetany due to a lowered calcium concentration) but not presumably because of a lack of readily oxidizable carbohydrate pabulum.

It might be suggested that in our animals showing hypoglycemic convulsions there may be no upset in the metabolism of the nervous system proper because, as already indicated, fructose may be an adequate substitute for glucose in such tissue, but that the initial irritation arises in some other tissue (e.g., end organs) unable to oxidize fructose and dependent upon a sufficient supply of glucose.

SUMMARY

The ability of the mammalian organism to utilize fructose in the absence of the liver has been confirmed. It has further been demonstrated that the life of the eviscerated animal can also be prolonged for a considerable time by administration of fructose, conclusively showing that in the absence of the liver and intestinal tract fructose is assimilated by an animal. In neither the hepatectomized nor the eviscerated animal was there an increase in the blood glucose following a large injection of fructose. This finding lends no support to the view that the fructose is first converted to glucose before utilization by muscle and brain and presumably by other tissues also, rather the experimental facts find a ready interpretation on the basis that in mammals fructose can be directly oxidized.

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IN VITRO ACTION OF CRYSTALLINE VITAMIN B_1 ON PYRUVIC ACID METABOLISM IN TISSUES FROM POLYNEURITIC CHICKS[1]

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As a result of investigations which were started in 1929 by Kinnersley and Peters, the conclusion was recently reached (1) that vitamin B_1 is indirectly concerned with the oxidative removal of lactate and pyruvate in avitaminous pigeon brain. It appeared that the vitamin catalyzed the coupled oxidation of some unknown substance in the presence of lactate or pyruvate. However, it could not be determined from their work which was immediately concerned in the interaction. In similar studies carried out in our laboratory on polyneuritic chicks, we (2) were unable to show any relationship between vitamin B_1 and lactate oxidation in brain tissue. In avitaminous heart tissue, on the other hand, an indirect relationship was indicated. By means of oxygen uptake studies it was shown that avitaminous heart had a subnormal ability to respire in lactate. Lactic acid analyses showed a decreased rate of lactate removal by avitaminous heart. Additions of vitamin B_1 had a stimulating effect upon the rate of respiration. It appeared, however, that this lowered ability of certain tissues from polyneuritic chicks to metabolize lactate was a secondary effect of the avitaminosis. It was found that additions of small amounts of pyruvate to heart and kidney exerted an inhibitory influence upon lactic acid dehydrogenase activity. This inhibition was especially marked in avitaminous tissues. It was demonstrated by Thompson and Johnson (3) that bisulphite-binding substances increase in the blood stream of pigeons and rats during polyneuritis. Recently Johnson (4) has succeeded in isolating the bisulphite-binding substance in the form of the 2:4 dinitrophenylhydrazone from the blood of B_1 deficient pigeons in sufficient quantities for characterization and has shown it to be pyruvic acid.

In the present communication results are presented from oxygen uptake studies upon tissues from normal and polyneuritic chicks using pyruvate as substrate. Additions of vitamin B_1 to avitaminous tissues respiring in pyruvate substrate were made. Results from chemical determination of

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the pyruvate removed by normal and avitaminous tissues and by avitaminous tissues with added vitamin are given. We find that in avitaminous cerebrum and kidney there is a decreased rate of respiration in pyruvate substrate. This lowered oxygen uptake was brought back to a more nearly normal level by the addition of crystalline vitamin B_1. Measurement of pyruvate removal shows a lowered rate of removal in avitaminosis. The *in vitro* addition of vitamin B_1 increased the removal of pyruvate in avitaminous tissue.

METHOD. The techniques employed in the production of acute polyneuritis in the experimental chicks and in the subsequent removal of tissues was identical with the methods we have previously described. The amount of minced tissue introduced into the Barcroft flasks was in all cases 0.150 to 0.160 gram for cerebrum, and 0.110 to 0.120 gram for kidney. The amount of tissue was kept uniform because the relative concentration of pyruvate with respect to tissue was found to be of importance, especially when studying pyruvate removal. Ringer phosphate-pyrophosphate buffer pH 7.3, which was M/30 in phosphate (Na) and M/100 in pyrophosphate (Na), was used throughout. The pyruvic acid was obtained from the Eastman Kodak Company and was freshly distilled in vacuo (37–40°C.) for use as substrate after being adjusted to pH 7.3 with NaOH. Concentrations of substrate are given in all experiments. The vitamin used in these studies was Merck's crystalline vitamin B_1.

An aqueous solution of the crystals was made to contain 25 gamma per cubic centimeter, and 0.1 cc. aliquots containing 2.5 gamma were added directly to the respirometer flasks containing minced tissue, buffer, and substrate to make a total volume of 3.0 cc. In earlier studies we used a vitamin B_1 concentrate (Oryzanin Fortior "Sankyo"). We have found these two vitamin preparations to be similar both in antineuritic activity and in their *in vitro* action. For the chemical determination of pyruvate removal by chick tissues the method of Clift and Cook (5), which depends upon the ability of sodium bisulphite to form an addition compound with pyruvic acid in acid solutions, was adopted. Results from this method have been shown by Peters and Thompson (6) to be in good agreement with results obtained using a modification of the Case 2:4 dinitrophenylhydrazone method (7) when pyruvic acid is used as substrate. Although there are other bisulphite-binding substances in biological materials besides pyruvic acid, this method affords a satisfactory means of following the removal of added pyruvate by tissues.

Determination of pyruvate removal by chick tissues. After the tissues had been aerobically shaken for two hours at 37°C. in the Barcroft differential manometers, the contents of the flasks containing tissues with no added pyruvate, with added pyruvate, and pyruvate plus vitamin were transferred to 5 cm. mortars to which had previously been added 1 cc. of 20 per

cent trichloracetic acid. At the same time the contents of two control flasks containing the same amount of pyruvate as had been added to the experimental flasks, but no tissue, were washed into 15 cc. graduated tubes containing 2 cc. of 20 per cent trichloracetic acid, made to volume, and later analyzed with the others in order to determine the original concentration of added pyruvate in the experimental flasks. The tissues were thoroughly ground in the small mortars with glass pestles and washed into 15 cc. graduated centrifuge tubes. After diluting to volume and mixing, the tubes were allowed to stand for one-half hour and then centrifuged. Aliquots from the centrifugates were transferred to 50 cc. Erlenmeyers, brought to pH 2.0 with sodium hydroxide, and treated with sodium bisulphite. It was found that further washing of the tissue residue with trichloracetic acid was unnecessary. The bisulphite-binding capacity was then determined by titration with N/200 iodine according to the procedure of Clift and Cook (5).

(1 ml. N/200 I = 0.22 mgm. pyruvic acid)

Results. It was found that pyruvate was readily utilized as substrate for respiration both by normal and polyneuritic chick cerebrum. Although the no-substrate level of respiration is not significantly reduced in avitaminous cerebrum, as may be seen in table 1, the increase in oxygen uptake produced by adding pyruvate was not as great in avitaminous as in normal cerebrum. This subnormal rate of respiration in the vitamin B_1 deficient cerebrum was brought back nearly to the normal level by the *in vitro* addition of 2.5 gamma of crystalline vitamin B_1. The increased oxygen uptake produced by the added vitamin is especially significant in the second and third 40 minute periods of respiration. In the absence of added vitamin the respiration of avitaminous cerebrum in pyruvate substrate steadily falls off during the course of the experiment. But when added vitamin B_1 is present the respiration of cerebrum in pyruvate is maintained at a level which approaches that of normal tissue.

In working with kidney special precautions had to be taken to use highly purified pyruvic acid. When a neutralized solution of pyruvate which had stood for over a week at 0° was tested, marked loss in bisulphite-binding capacity, sometimes as much as 40 per cent, was observed. Such pyruvate, when used as substrate for avitaminous kidney in concentrations greater than 0.005M produced definite inhibition of respiration, and additions of vitamin B_1 brought back the lowered respiration to a no-substrate level. Impure pyruvate had no effect upon normal kidney respiration. These results, which have been repeatedly observed, indicate that pyruvic acid containing appreciable amounts of higher polymers is unsuitable for use as tissue substrate. The impurities of pyruvic acid are especially toxic to avitaminous kidney and are rectified by additions of vitamin B_1. How-

TABLE 1

Effect of vitamin B_1 upon the oxygen uptake of avitaminous cerebrum as compared with normal cerebrum in pyruvate

P = pyruvate; V = vitamin B_1 2.5 gamma. Expressed as cmm. O_2/gm./hr. (wet weight) (N. T. P.).

EXPERIMENT	SUBSTRATE	CONCENTRATION OF PYRUVATE IN RESPIROMETERS	PERIODS (MINUTES)			TOTAL CCM GM. 2 HRS
			0-40	40-80	80-120	
Avitaminous cerebrum						
100	—	—	930	751	597	1,519
	P	0.012 M	1,410	1,257	1,023	2,460
	P + V	0.012 M	1,638	1,451	1,268	3,015
101	—	—	1,125	771	551	1,632
	P	0.017 M	1,609	1,188	885	2,460
	P + V	0.017 M	1,666	1,420	1,322	2,781
102	—	—	996	582	447	1,330
	P	0.016 M	1,522	1,098	1,030	2,440
	P + V	0.016 M	1,717	1,213	1,226	2,800
103	—	—	871	691	586	1,419
	P	0.017 M	1,551	1,285	1,057	2,599
	P + V	0.017 M	1,470	1,468	1,217	2,772
104*	—	—	1,000	760	546	1,540
	P	0.015 M	1,720	1,410	960	2,797
	P + V	0.015 M	1,690	1,593	1,125	2,926
Ave.	—		984	711	545	1,488
	P		1,562	1,247	991	2,551
	P + V		1,636	1,429	1,232	2,859
Normal cerebrum						
105	—	—	1,197	792	615	1,735
	P	0.011 M	1,670	1,604	1,377	3,246
106	—	—	996	709	477	1,448
	P	0.021 M	1,769	1,665	1,510	3,300
107	—	—	625	684	552	1,350
	P	Not determined	1,756	1,620	1,358	3,105
108	—	—	940	812	517	1,524
	P	0.032 M	1,840	1,734	1,545	3,425
Ave.	—		939	749	540	1,514
	P		1,758	1,656	1,448	3,294

(Ringer's phosphate-pyrophosphate used throughout.)

* Mild case of polyneuritis.

TABLE 2

Effect of vitamin B_1 upon the oxygen uptake of avitaminous kidney as compared with normal kidney in pyruvate

P = pyruvate; V = vitamin B_1 2.5 gamma. Expressed as cmm. O_2/gm./hr. (wet weight) (N. T. P.).

EXPERIMENT	SUBSTRATE	CONCENTRATION OF PYRUVATE IN RESPIROMETERS	PERIODS (MINUTES)			TOTAL, CMM. GM. 2 HRS.
			0–40	40–80	80–120	
Avitaminous kidney						
110	—	—	1,751	1,561	1,358	3,110
	P	0.0038 M	1,857	1,833	1,649	3,558
	P + V	0.0038 M	1,933	1,923	1,707	3,708
	P	0.0076 M	1,848	1,789	1,690	3,524
	P + V	0.0076 M	2,197	2,161	1,927	4,200
111	—	—	1,600	1,559	1,200	2,900
	P	0.0038 M	1,743	1,864	1,624	3,485
	P + V	0.0038 M	1,885	2,114	1,670	3,770
	P	0.0076 M	1,870	2,032	1,723	3,735
	P + V	0.0076 M	1,998	2,245	1,839	4,060
	P	0.015 M	1,614	1,747	1,488	3,241
112	—	—	1,479	1,479	1,201	2,778
	P	0.0076 M	1,400	1,475	1,270	2,760
	P + V	0.0076 M	1,652	1,808	1,571	3,350
113	—	—	1,827	1,503	1,387	3,140
	P	0.0042 M	1,581	1,441	1,398	2,938
	P + V	0.0042 M	1,962	1,729	1,719	3,615
Ave.	—		1,664	1,525	1,286	2,982
	P		1,718	1,739	1,559	3,333
	P + V		1,938	1,980	1,756	3,951
Normal kidney						
121	—	—	1,810	1,604	1,418	3,200
	P	0.0038 M	2,350	2,340	2,042	4,480
122	—	—	1,690	1,611	1,390	3,130
	P	0.0076 M	2,645	2,630	2,390	5,120
123	—	—	2,080	1,722	1,453	3,502
	P	0.0044 M	2,142	2,081	1,977	4,140
124	—	—	1,975	1,810	1,501	3,520
	P	0.015 M	2,532	2,446	2,404	4,970
Ave.	—		1,881	1,687	1,443	3,338
	P		2,417	2,374	2,203	4,678

ever, pyruvate from which the impurities were removed by repeated vacuum distillation was readily utilized by normal cerebrum and kidney. The respiration of kidney was markedly influenced by the concentration of pyruvate used. Maximum stimulation was produced by low concentrations of pyruvate. The effect of variations in pyruvate concentration is given in experiment 111, table 2. It may be seen that 0.0038M and 0.0076M pyruvate produced stimulation to oxygen uptake of avitaminous kidney; but when the pyruvate concentration was increased to 0.015M the oxygen uptake was only slightly greater than the no-substrate uptake. This same concentration of pyruvate was readily utilized by normal kidney as is shown by experiment 124. Thus, there is a lowered pyruvate tolerance in avitaminous kidney. Table 2 also shows that the lower concentrations of pyruvate are not readily utilized by avitaminous kidney. In some cases the addition of pyruvate produced no increase in avitaminous

TABLE 3

Effect of vitamin B_1 upon aerobic removal of added pyruvic acid by avitaminous cerebrum

Pyruvic acid removal is given as mgm./gm. fresh tissue/2 hrs.

EXPERIMENT	INITIAL CONCENTRATION, MGM./GM. TISSUE	ACTUAL REMOVAL			THEORETICAL REMOVAL EFFECT OF VITAMIN (FROM EXTRA O_2 UPTAKE)
		Without vitamin	With vitamin	Effect of vitamin (difference)	
109	32.0	11.21	15.21	4.00	0.04
101	28.4	7.23	10.78	3.55	0.42
103	27.8	6.11	6.99	0.88	0.27
102	27.0	3.98	5.90	1.92	0.57
104*	25.3	6.19	6.77	0.58	0.21
100	20.2	3.61	7.81	4.20	0.87

* Mild case of polyneuritis.

kidney respiration. However, the oxygen uptake of normal kidney was greatly increased by pyruvate additions. When 2.5 gamma of vitamin were added to avitaminous kidney its respiration in pyruvate substrate was always improved, but was not restored to the normal level. We have also made additions of vitamin to avitaminous kidney in the absence of added substrate, but obtained no significant increases in oxygen uptake. Likewise, additions of vitamin B_1 to normal kidney with and without added pyruvate were without effect.

In order to see whether the extra oxygen uptake obtained with added vitamin B_1 was accompanied by an increased removal of pyruvate, the method of Clift and Cook (5) was applied to the contents of the respirometer flasks after shaking for two hours. The method followed in the treatment of the tissues for analysis is described above. The results obtained with avitaminous cerebrum are given in table 3.

It will be seen that with the exception of experiment 104, in which case the chick exhibited only mild symptoms of polyneuritis, the removal of pyruvate was proportional to the concentration of the pyruvate. In all cases the amount of pyruvate removed by avitaminous cerebrum was increased by adding vitamin B_1 to the tissue. Variations in the amount of pyruvate removed by the vitamin appear to be due to variations in the severity of the polyneuritis. Although we never use chicks in these studies until they exhibit head retractions and are unable to walk, there are still variations depending upon the length of time they have been in this condition. A greater vitamin effect is always obtained with a more severe case of polyneuritis. The theoretical amount of pyruvate oxidatively removed by the vitamin was calculated from the extra oxygen uptake produced by

TABLE 4

Effect of vitamin B_1 upon aerobic removal of added pyruvic acid by avitaminous kidney

Pyruvic acid removal is expressed as mgm./gm. fresh tissue/2 hrs.

EXPERIMENT	INITIAL CONCENTRATION, MGM./GM. TISSUE	ACTUAL REMOVAL			THEORETICAL REMOVAL. EFFECT OF VITAMIN (FROM EXTRA O_2 UPTAKE)
		Without vitamin	With vitamin	Effect of vitamin (difference)	
115	38.8	6.35	6.73	0.38	0.43
117	32.0	6.08	8.26	1.18	0.76
116	15.5	6.58	7.29	0.71	0.84
113	10.0	5.01	6.27	1.26	1.06
114	7.6	3.70	4.45	0.75	0.45

the vitamin. The assumption is made that the pyruvate is completely oxidized to CO_2 and H_2O according to the following equation:

$$CH_3COCOOH + 5/2\ O_2 \rightarrow 3\ CO_2 + 2H_2O$$

The extra oxygen uptake was in no case sufficient to account for all of the pyruvate removed. It was thought that there might be some loss of pyruvate due to an acid-stable combination of its carbonyl group with some binding group present in the tissue; but experiments run under anaerobic conditions showed that there was no removal of added pyruvate in the absence of oxygen by either normal or avitaminous cerebrum and kidney. Lactic acid determinations have also been made upon tissues with added pyruvate with and without added vitamin, but no indications for a reduction of pyruvic acid to lactic acid were obtained. It is probable that the pyruvate is incompletely oxidized or a portion of it is removed by aerobic synthesis.

Increased removal of pyruvate by added vitamin was also found in avitaminous kidney as is shown in table 4. But in kidney better agreement

was obtained between the actual and the theoretical effect of the vitamin upon pyruvate removal.

Discussion. Although these experiments with polyneuritic chick cerebrum and kidney demonstrate that vitamin B_1 is concerned in the oxidative metabolism of pyruvic acid, the elucidation of the exact stage of its oxidation effected by vitamin B_1 and the mechanism of the reaction await further investigations. A comparison of these results with our earlier results obtained with lactic acid substrate indicates that in chicks the vitamin is more closely associated with pyruvate than with lactate, since we were unable to obtain any vitamin effect in cerebrum using lactate substrate. With pyruvate substrate vitamin effects in cerebrum as well as kidney were consistent. In pigeon brain Peters and his co-workers have obtained an effect of the vitamin upon respiration both in lactate and pyruvate, and have found the two acids interchangeable. However, Meikeljohn (8) showed that the extra oxygen uptake produced by adding vitamin to pigeon cerebrum in lactate substrate was not accompanied by an increased removal of lactate. It appears that the vitamin is concerned with pyruvate removal, and that apparent vitamin effects upon respiration in lactate substrate may be secondary to its action in the removal of pyruvate, since small amounts of pyruvate have been shown to exert an inhibitory action upon lactic acid dehydrogenase activity of tissues. However, the fact that the addition of vitamin B_1 alone does not completely restore the utilization of pyruvate by avitaminous tissue to a normal level suggests that either there is a loss of some other component besides vitamin B_1 necessary for pyruvate metabolism, or that the effects here observed are still secondary to the participation of the vitamin in some other reaction closely associated with pyruvate oxidation.

SUMMARY

1. The oxygen uptake of minced cerebrum and kidney from normal and polyneuritic chicks in pyruvate substrate was studied. The effect of added vitamin B_1 upon pyruvate oxidation in avitaminous cerebrum and kidney was determined by means of oxygen uptake studies and chemical determinations of pyruvate removal.

2. Respiration of vitamin B_1 deficient cerebrum in pyruvate is subnormal. The ability of chick kidney to utilize pyruvate substrate for respiration is seriously impaired in polyneuritis.

3. The oxygen uptake of avitaminous cerebrum and kidney in pyruvate substrate is increased to a nearly normal level by *in vitro* additions of small amounts (2.5 gamma) of vitamin B_1.

4. This extra oxygen uptake produced by added vitamin B_1 to avitaminous tissues is accompanied by an increased removal of pyruvate.

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A STUDY OF ANAEROBIC GLYCOLYSIS IN TISSUES FROM POLYNEURITIC CHICKS[1]

The Negative Action of Vitamin B_1

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It has been demonstrated in previous publications (1, 2) that in polyneuritic chicks there are abnormalities in the oxidative metabolism of lactic and to a greater extent of pyruvic acid which can be largely alleviated by the *in vitro* addition of vitamin B_1 to the avitaminous tissues. Although these results can all be adequately explained on the basis that vitamin B_1 functions in the oxidative removal of these intermediates in carbohydrate breakdown, it seemed possible that in the absence of vitamin B_1 there might also be abnormalities in anaerobic glycolysis of tissues. Although Neuberg and his school advocate methylglyoxal as an intermediate in the formation of lactic acid from carbohydrate, Embden and his co-workers (3) and Meyerhof and Keissling (4) found that in muscle pyruvic acid is the precursor of lactic acid.

Geiger (5), in a study of the rôle of glutathione in anaerobic tissue glycolysis, suggests that lactic acid production from glucose may pass through the intermediate methyglyoxal, but that the breakdown of glycogen to lactic acid follows the Embden-Meyerhof scheme which involves the intermediate pyruvic acid. He found that reduced glutathione was essential in the conversion of glucose and methylglyoxal into lactic acid, but not in the breakdown of glycogen to lactic acid.

Anaerobic glycolysis was, therefore, studied in polyneuritic chick tissues with and without added vitamin B_1 in order to determine whether or not the vitamin was necessary in the anaerobic formation of lactic acid. We were primarily interested in determining the amount of pyruvic and lactic acid which accumulated under these conditions.

The results obtained from these experiments show that avitaminous tissues readily form lactic acid (from glucose and glycogen) under anaerobic conditions and that added vitamin B_1 is not necessary in the anaerobic conversion of pyruvic acid into lactic acid.

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METHODS. Tissues from large polyneuritic chicks (200–400 grams) were used in these studies. The chicks were killed by decapitation and the organs to be studied were immediately removed and placed upon filter papers where they were carefully wiped free from excess blood. The tissues were then placed upon watch glasses and finely minced with scissors. Portions of the tissue mince were weighed on pieces of cover slip and introduced into Thunberg tubes containing 4 cc. of buffer solution (ringer phosphate

TABLE 1

Anaerobic glycolysis of B_1 deficient chick tissues. The negative effect of added vitamin B_1

TISSUE	BUFFER	SUBSTRATE	BISULPHITE-BINDING SUBSTANCES CALCULATED AS MGM. PYRUVIC ACID PRODUCED BY 100 GRAMS TISSUE PER HOUR		MGM. LACTIC ACID PRODUCED BY 100 GRAMS TISSUE PER HOUR	
			No vitamin	Added vitamin	No vitamin	Added vitamin
Cerebrum	Ringer phosphate		2.5	2.5 (4γ)	32	31 (4γ)
Cerebrum	Ringer phosphate	Glucose 12 mgm.	3	3 (4γ)	322	320 (4γ)
Heart	Ringer phosphate		2	2.5 (4γ)	50	50 (4γ)
Heart	Ringer phosphate	Glucose 12 mgm.	4	3.5 (4γ)	205	199 (4γ)
Kidney	Ringer phosphate	Glucose 12 mgm.	13	15 (4γ)	190	211 (4γ)
Skeletal muscle	Ringer phosphate	Glucose 12 mgm.	1.5	2 (4γ)	275	290 (4γ)
Heart	Ringer phosphate	Glycogen 60 mgm.	6	5 (2γ)	156	156 (2γ)
Kidney	Ringer phosphate	Glycogen 60 mgm.	15	14 (2γ)	141	149 (2γ)
Liver	Ringer phosphate	Glycogen 60 mgm.	23	22 (2γ)	68	68 (2γ)
Skeletal muscle	Ringer phosphate	Glycogen 60 mgm.	11	10 (2γ)	340	339 (2γ)
Heart	Ringer phosphate + pyrophosphate	Glycogen 60 mgm.	5	5 (5γ)	149	153 (5γ)
Kidney	Ringer phosphate + pyrophosphate	Glycogen 60 mgm.	18	19 (5γ)	159	156 (5γ)
Liver	Ringer phosphate + pyrophosphate	Glycogen 60 mgm.	16	19 (5γ)	65	79 (5γ)
Skeletal muscle	Ringer phosphate + pyrophosphate	Glycogen 60 mgm.	9	10 (5α)	482	494 (5γ)

± pyrophosphate), pH 7.3. A measured amount (1 cc.) of an aqueous solution of substrate was pipetted into the bulb of the Thunberg tubes. The vitamin B_1 concentrate used was Oryzanin Fortior "Sankyo" obtained from the Takamine Corporation. The vitamin solution replaced an equivalent amount of buffer solution in the tubes to which vitamin additions were made. The tubes were then evacuated and placed in a constant-temperature bath maintained at 37°C. The substrates were mixed with

the tissues and incubated at 37°C. for a definite length of time, during which the tubes were frequently shaken by hand. At the end of the incubation period the tubes were opened and 2 cc. of 20 per cent trichloracetic acid were added immediately. The contents of the tubes were then washed into small mortars where the tissue particles were thoroughly ground. The solutions were then transferred to 15 cc. centrifuge tubes, made to volume, and centrifuged. Measured aliquots were analyzed for pyruvic acid according to the method of Clift and Cook (6) and for lactic acid according to the method of Friedemann and Graeser (7).

The glycogen used in these experiments was prepared from fresh beef liver according to the method of Sahyun and Alsberg (8). This method of preparation is rapid, requires no alkaline digestion, and yields a product of excellent quality.

Results. The values obtained with various chick tissues are shown in table 1. The results show that avitaminous tissues readily form lactic acid from glucose and glycogen under anaerobic conditions. Vitamin B_1 added to avitaminous tissues has no apparent effect upon tissue glycolysis. There is no evidence of an accumulation of pyruvic acid under these conditions. It is of interest to note that of the tissues studied, kidney and liver have by far the highest content of bisulphite-binding substances. In these organs, values of 15 to 20 mgm. pyruvic acid/100 gm./hr. were found, whereas in other organs the values range from 2 to 5. Skeletal muscle appears to be intermediate in this respect. But the addition of vitamin B_1 has no effect upon this phenomenon.

Discussion. These experiments show that anaerobic glycolysis readily takes place in tissues from polyneuritic chicks. Abnormalities in pyruvate metabolism, which have been previously described and shown to be closely associated with vitamin B_1, cannot be attributed to faulty glycolysis in avitaminosis B_1. There is no evidence that vitamin B_1 has a function in anaerobic carbohydrate metabolism. Its action appears to be limited to the oxidative metabolism of these carbohydrate intermediates.

SUMMARY

1. Anaerobic glycolysis was studied in vitamin B_1-deficient chick cerebrum, heart, kidney, liver, and skeletal muscle. The effect of *in vitro* additions of vitamin B_1 upon the anaerobic formation of lactic and pyruvic acid from glucose and glycogen was determined.

2. Avitaminous tissues readily form lactic acid from glucose and glycogen under anaerobic conditions.

3. There is no significant accumulation of pyruvic acid anaerobically.

4. Added vitamin B_1 has no effect on anaerobic glycolysis.

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FURTHER STUDIES ON THE EFFECTS OF NaF ADMINISTRATION UPON THE BASAL METABOLIC RATE OF EXPERIMENTAL ANIMALS[1]

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The level of administration of NaF in the studies reported by Phillips et al. in 1935 was three or more times that suggested for the control of thyrotoxicosis by Goldemberg in 1932. It seemed that the level of feeding used might have been too high to produce the reduction in the metabolic rate claimed by Goldemberg. Since the previous studies did not duplicate the exact dosage used by Goldemberg, a second series of studies was undertaken with a view of extending the results previously obtained.

Accordingly, an experiment was planned to determine the effects of the mode of administration, the dosage, and the element of time in an attempt to influence the metabolic rate of experimental animals by the use of NaF. Further, it seemed advisable to determine if previous sensitization to desiccated thyroid influenced the subsequent action of NaF. Data were also desirable in more than a single species. Guinea pigs were employed in one experiment and data were obtained as in the case of the rat with the additional study of the effects of scurvy on the BMR.

EXPERIMENTAL. Vigorous mature male rats were used in one experiment. As in the former case, the basal ration A described by Lamb et al. in 1933 was used. When fluorine was given it was injected intraperineally in a solution whose concentration was 0.9 per cent and at a rate of 18 mgm. of NaF per kilogram of body weight. This was the concentration and dosage favorably reported by Goldemberg in 1930 for the reduction of the normal metabolic rate of experimental animals. In the case of the guinea pigs fairly mature but still growing animals were used. A basal ration composed of rolled oats 68 parts, artificially dried alfalfa hay 20 parts, commercial casein 5 parts, yeast 5.5 parts, irradiated yeast 0.5 part, steamed bone meal 1 part, and iodized NaCl 0.5 was fed. This ration gave excellent results when supported by ample daily doses of orange juice (5 cc.) and it produced scurvy in 28 days when orange juice was withheld.

In each experiment the basal metabolic rates were obtained several times for each animal before subjecting it to experimental treatment. Thus, a

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normal BMR was obtained for each individual. Subsequently the experiment was so arranged as to permit a weekly basal metabolism determination for each animal throughout each phase of the study. In this manner the performance of a single animal was directly comparable to its own normal record as well as to the record of any control animals throughout the experiment. The basal metabolic rates were determined as outlined by Phillips et al. in 1935. The body weights were recorded.

Results. The body weights of the experimental animals present interesting results. Fully mature male rats weighing 350 to 400 grams each lost 15 to 30 per cent of their body weight when subjected to a weekly fast of 24 hours, duration and a ration containing 0.25 per cent desiccated thyroid. Withdrawal of the desiccated thyroid from the ration caused a prompt and steady increase in body weight until the original body weights were regained. The intraperitoneal injection of a single dose of NaF (18 mgm./kgm.) during the middle of the third week following the removal of the desiccated thyroid promptly arrested body weight recovery and caused a sharp loss almost equivalent to that lost during the desiccated thyroid feeding period. In younger male rats (250 grams) little loss of weight was caused by feeding 0.25 per cent desiccated thyroid in the ration. However, a single intraperitoneal injection of NaF during the third week after cessation of desiccated thyroid feeding produced the same response as recorded for the older and heavier animals. The continuous daily intraperitoneal injections of 18 mgm. of NaF per kilogram of body weight and a weekly 24 hour fast caused a variable reaction upon body weight during the first week. Thereafter there was a gradual and continuous loss in body weight, amounting to 14 or 15 per cent in 28 to 35 days at which time the injections were discontinued.

The intraperitoneal injection of NaF (18 mgm./kgm.) in the guinea pigs seemed to have little influence upon growth. When the same animals were changed from a daily injection of 18 mgm. of NaF per kilogram of body weight to the administration of an oral dose of 50 to 60 mgm. of NaF fed in solution by pipette, growth was arrested during the third week. A sharp and rapid loss of body weight occurred, which amounted to 35 or 40 per cent. The weight records of the scorbutic animals were typical.

Table 1 shows the effect of NaF administration upon the basal metabolic rate. The method used for making the BMR determinations has repeatedly given consistent duplicate results with a variability within ± 7 per cent. A variation of 10 per cent should permit a conservative limit for comparison of results between weekly determinations. With this as a guide it seems that continuous daily injection of 18 mgm. of NaF per kilogram of body weight may cause a slight rise in the metabolic rate of the rat in 28 days. No change was noted in the case of the guinea pig. When their dose of NaF was changed to an oral dose of 50 to 60 mgm. of NaF per

kilogram of body weight no pronounced change was noted although a tendency to increase was indicated by the 28th day. The route of administration or the dosages used had no effect upon the normal metabolic rate of these animals.

TABLE 1

The effect of NaF administration upon the basal metabolic rate

(Cal. per sq. m. of body surface per day)

	RAT 1	RAT 2	GUINEA PIG 1	GUINEA PIG 2
	Basal rate			
	694	640	775	754
NaF injected intraperitoneally (18 mgm./kgm. of body weight)				
2–4 hours	580		650	732
7th or 14th day	770	623	795	690
7th–14th day 2 hours after injection	727	600	856	775
28th day	806	746	785	815
NaF fed (50–60 mgm./kgm. of body weight)				
14th day			710	607
21st day			728	608
28th day			859	

TABLE 2

The effect of previous desiccated thyroid feeding upon the reaction to fluoride administration

(Cal. per sq. m. of body surface)

	RAT 1	RAT 2	RAT 3	RAT 4	RAT 5	RAT 6
	Basal rate					
	634	718	722	710	828	855
Desiccated thyroid feeding (0.25 per cent level)						
14th day	1,280	1,576	1,365	1,640	610	1,400
28th day	1,415	875	2,015	2,000		
Returned to basal ration without desiccated thyroid						
21st day	744	614	783	970	750	740
21st day—2 hours after NaF injection	985	945			750	832
55th day		670	792	705		756
55th day—2 hours after NaF injection		605	705	645	705	730

Table 2 shows that the feeding of 0.25 per cent of desiccated thyroid to the rat causes an increase in the metabolic rate by the 14th day of feeding and this generally continued to mount to the 28th. In this experiment the BMR had returned to normal 21 days after the end of the 4-week desic-

cated thyroid feeding period. At this time the basal rates were distinctly raised by the intraperitoneal injection of NaF in 3 cases out of 4. This indicates that a residual desiccated thyroid effect was still present. This reaction was lost by the 55th day following the removal of desiccated thyroid from the ration. The results seem to indicate a small and uniform lowering of the basal metabolic rates of all of the animals on the 55th day following the injection of NaF. These results, however, lie within the limits of ± 10 per cent of their original basals and are unlikely to be of special significance. The data in table 2 are interpreted to mean that the administration of NaF does not lower the basal metabolic rate of the rat following the feeding of desiccated thyroid once the effect of the latter has completely vanished. NaF seems to augment the action of the desiccated thyroid so long as a residual sensitization to desiccated thyroid is present

TABLE 3

The effect of scurvy upon basal metabolism

(Cal. per sq. m. of body surface)

	ANIMAL 3	ANIMAL 4	
	Basal rate		
	812	780	
	Scurvy ration		
14th day	780	743	
21st day	773	680	Growth arrested
28th day	859	971	Typical scurvy
35th day	725	830	After 10 cc. of O. J. on 28th day
35th day	960	825	2 hours after NaF injection
42nd day	778	740	5 cc. of O. J. on 35th day
54th day	786	800	Severe chronic scurvy

in the body. Feeding 0.20 per cent desiccated thyroid caused death in two guinea pigs on the 7th day.

In table 3 the effects of the development of scurvy and the basal metabolic rate are recorded. There is a tendency for the basal metabolic rate to increase when the deficiency approaches the stage of typical scorbutic symptoms. It is to be noted that a definite increase is recorded in both cases. In one of these cases the increase exceeded the original basal rate by more than 10 per cent. An attempt was made to maintain these animals and obtain another record in typical scurvy but it was unsuccessful. The feeding of single doses of orange juice caused the basal metabolic rate to resume its normal level. When NaF was injected intraperitoneally on the 35th day when the animals were in sub-acute scurvy, variable results were secured. A rise was recorded in one case and none in the other.

SUMMARY AND CONCLUSIONS

The administration of NaF has been found to be without effect upon the basal metabolic rate in the normal experimental animal. No difference in action was noted regardless of whether 18 mgm. of NaF per kilogram of body weight were injected intraperitoneally or were fed orally at the rate of 50 to 60 mgm. of NaF per kilogram of body weight. Thus, the route of administration or the dosage has no influence upon the results obtained. Time, as evidenced by daily intraperitoneal injections, was without influence from 2 hours after the injection up to 28 days, although in the latter case a slight increase was recorded. Previous sensitization with desiccated thyroid failed to produce an effect after 21 days. NaF injections during the period of desiccated thyroid sensitivity caused a rise in the metabolic rate.

The metabolic rate tended to rise in the condition of scurvy, or when NaF was injected in sub-acute scurvy. The results were variable and inconclusive.

The daily intraperitoneal injection of a solution of NaF at the rate of 18 mgm. per kilogram of body weight caused a loss of body weight in the rat which amounted to 14 or 15 per cent. This was closely parallel to the loss of body weight caused by feeding 0.25 per cent of desiccated thyroid. The body weight of guinea pigs was not greatly influenced by the level of 18 mgm. of NaF injected daily, but was sharply reduced by feeding 50 to 60 mgm. of NaF per kilogram of body weight. Two-tenths per cent desiccated thyroid proved fatal to guinea pigs in 7 days.

These results with rats and guinea pigs do not confirm the published work of Goldemberg. Insofar as previous sensitization to desiccated thyroid simulates a condition of thyrotoxicosis, there is no evidence from these experiments to indicate that it could be controlled by NaF feeding. Neither has it been possible to reduce the normal metabolic rate of experimental animals by NaF administration under a variety of conditions.

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THE RELATION OF PANCREATIC JUICE TO PANCREATIC DIABETES[1]

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Ever since the discovery by von Mering and Minkowski (1890) that extirpation of the pancreas in the dog produces a condition closely resembling diabetes mellitus in man, reports have appeared in the literature implicating the external secretion of the pancreas in this condition. In the intervening years much evidence has accumulated which indicates that the acinar tissue and presumably its product, pancreatic juice, play no rôle in pancreatic diabetes, but that this condition is due to partial or complete insufficiency of islet function. This evidence need not be reviewed here. There appears to be substantial basis for the generally accepted view that insulin is a product of the pancreatic islets and probably specifically of the beta cells. The uncertainty rests with the possible inter-relationship between insulin and the external secretion, the involvement of the latter in human diabetes, and in the mechanism of insulin action. A diminution in the enzymatic activity of the pancreatic juice in human diabetes has been reported by Katsch and von Friedrich (1922), Jones, Castle, Mulholland and Barley (1925), Labbé, Nepveux, and Adlersberg (1925), and Gavrila and Paraschivesco (1926). La Barre and Destree (1928) and La Barre (1930) demonstrated by means of cross circulation experiments, in which the blood of the donor was circulated through the head only of the recipient, that hyperglycemia in the donor increased the volume and enzyme concentration of the pancreatic secretion in the recipient. Conversely hypoglycemia in the donor diminished the secretion of pancreatic juice in the recipient. Baxter (1932) reported that in rabbits the injection of insulin produced hypoglycemia and diminished the output of enzymes in the pancreatic juice. Conversely Babkin (1935) found that in the same animal hyperglycemia increased the output of enzymes in the pancreatic juice, the effect being upon the endings of the parasympathetic nerves since it was abolished by atropine.

W. N. Boldyreff and E. B. Boldyreff (1928) have proposed a novel theory of pancreatic diabetes which implicates the external secretion. This

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theory may be stated somewhat as follows. Insulin, absorbed into the blood, stimulates the secretion of pancreatic juice. This juice contains a glycolytic substance and its absorption from the small intestine is responsible for the insulin effect on the blood sugar. In support of this theory W. N. Boldyreff (1934) states that when he ligated the smaller pancreatic duct of the dog and used the larger one for an external fistula, the symptoms of diabetes appeared in 24 hours. When the ligature was removed from the smaller duct the severe diabetic symptoms decreased at once and when the larger duct was closed the animal completely recovered. "Therefore," he concludes, "removal from the animal or human body of the external secretion leads to diabetes, hence the direct conclusion to treat diabetes by the introduction of pancreatic juice." E. B. Boldyreff (1928) reports that "during the periods of pancreatic secretion the sugar content is lower than during intervals between periods" and "pancreatic juice secreted into the small intestine and absorbed by the blood is one of the chief factors responsible for the changes in blood sugar content."

The repeated observations in many physiological laboratories that the production of a pancreatic fistula in the dog does not cause glycosuria and diabetes might be thought to have settled this question. However, it must be conceded that the customary methods for making such fistulae in the dog do not deprive the animal of all pancreatic juice because of the uniform presence of one or more accessory ducts. The effect of feeding pancreatic juice on the course of pancreatic diabetes has not been adequately tested, probably because of the difficulty in securing sufficient amounts of the fresh secretion. The administration of the whole fresh gland or of artificial juice made by extracting the fresh gland does not answer the question since these products contain materials which are not present in pancreatic juice. The development by L. R. Dragstedt, Montgomery, and Ellis (1930) of a method for making a total external fistula of all pancreatic ducts in the dog and for collecting the entire secretion provided an opportunity for reinvestigating this supposed relation of pancreatic juice to pancreatic diabetes.

EXPERIMENTAL PROCEDURE. Two types of experiment were done. In one group of animals the effect of the total loss of pancreatic juice on the development of diabetes was observed, while in the other an examination was made to determine the effect of the oral administration of pancreatic juice after complete pancreatectomy. Complete external fistulae of the pancreatic ducts were made in dogs in the following manner. An isolated closed sac was prepared of that portion of the duodenum, immediately below the entrance of the bile duct, which receives the various pancreatic ducts. A gold-plated cannula was placed in this sac and led through a stab wound in the abdominal wall. The sac and cannula were carefully wrapped with omentum and an end to end anastomosis made between the proximal and distal duodenum (fig. 1). By this method all of the external

secretion of the pancreas was lost to the body but could be quantitatively collected and administered to other animals. The secretion was activated by the admixture of succus entericus provided by the short segment of duodenum. These animals secreted from 500 to 1,400 cc. of pancreatic juice per 24 hours on the standard diet. To counteract the effect of the extensive loss of base and to prevent death from dehydration and acidosis, daily intravenous injections of 700 to 1,000 cc. of Ringer's solution were given. Small amounts of calcium carbonate and sodium bicarbonate were administered daily by mouth to prevent the occurrence of gastric or duodenal ulcers (Matthews and L. R. Dragstedt, 1932). With these

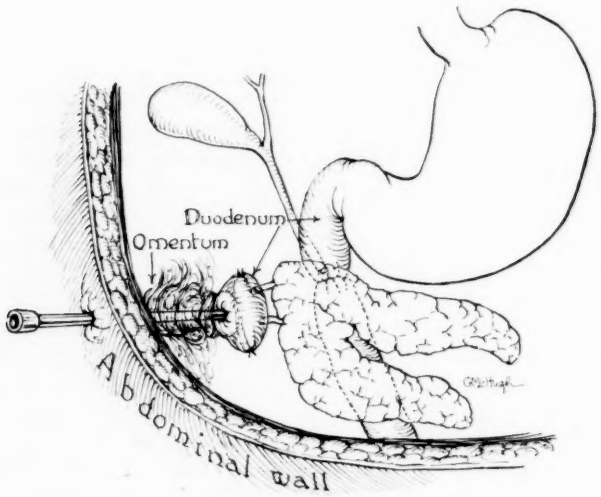

Fig. 1. Diagram of the method for making a total pancreatic fistula

precautions the animals, six in number, were kept in good condition for many weeks and provided a constant supply of active pancreatic juice. The urine of these dogs remained constantly free of sugar, except for an occasional sample usually immediately after operation when a trace of reducing substance was found. Three examinations of the fasting blood sugar in each animal were made at scattered intervals but all were well within the values found in normal dogs.

Healthy adult male dogs were completely depancreatized in a one-stage operation under ether anesthesia. After recovery each animal was placed on the standard diet consisting of 400 grams of meat, 400 cc. of whole milk, and 100 grams of white bread. Insulin was administered twice daily and an attempt was made to adjust the dosage so as to permit of only a

moderate glycosuria. The amount of sugar excreted in the urine was determined daily and occasional measurements were made of the blood sugar. Pancreatic juice was administered by stomach tube when not taken voluntarily. The freshly collected secretion only was used because of the well-known rapid deterioration of trypsin. In no instance was juice more than 24 hours old employed. The data on six depancreatized dogs are summarized in the following protocols.

PROTOCOLS. *Dog 1.* Weight 10.0 kgm. Pancreatectomy 1–31–35. After recovery the animal was placed on the standard diet and given 100 to 300 cc. of pancreatic juice daily. Fifteen to 18 units of insulin per day were given in three doses. During the first month the sugar excretion varied between 1.2 and 34 grams per day with an average of 6.4 grams. Conclusion: 300 cc. of pancreatic juice did not prevent or cure diabetes in this case.

Dog 2. Male. Weight 15.0 kgm. Pancreatectomy 2–1–35. After recovery this animal took the standard diet and from 75 to 300 cc. of pancreatic juice per day. During the first 18 days 15 units of insulin were given daily and the sugar excretion varied between 3 and 37 grams per day with an average of 13.5 grams. The insulin was then increased to 30 units and the sugar excretion decreased to an average of 5.1 grams per day for the following three weeks. Conclusion: 300 cc. of pancreatic juice did not prevent or cure diabetes in this case.

Dog 3. Male. Weight 6.7 kgm. Pancreatectomy 1–7–35. Standard diet and 200–500 cc. pancreatic juice daily. For the first month the daily sugar excretion averaged 7 grams on 15 to 20 units of insulin. Conclusion: 500 cc. of pancreatic juice did not prevent or cure diabetes in this case.

Dog 4. Male. Weight 16.8 kgm. Pancreatectomy 4–2–35. After recovery the animal took the standard diet and from 4–5–35 to 4–23–35 was given 500 to 800 cc. of pancreatic juice and 30 to 35 units of insulin. During this period the daily sugar excretion varied between 5 and 56 grams with an average of 11 grams. From 4–24–35 to 4–30–35 the juice was not given but the insulin and diet continued as before. During this period the daily sugar excretion varied between 2 and 11 grams with an average of 7.7 grams. From 5–1–35 to 5–19–35 from 600 to 800 cc. of pancreatic juice and 40 units of insulin were given daily and the sugar excretion averaged 9 grams. The juice was then withdrawn for 3 days and the sugar excretion promptly decreased, the urine becoming sugar-free on 5–22. On 5–23 and 5–24 there were severe hypoglycemia convulsions which were promptly relieved by glucose. From 5–28 to 6–16 from 800 to 1100 cc. of pancreatic juice and 40 units of insulin were given daily and the sugar excretion varied between 5 and 50 grams with an average of 26 grams per day. No juice was then given from 6–17 to 6–26 but the diet and insulin continued as before. The sugar excretion varied between 0 and 30 grams with an average of 8 grams.

Dog 5. Male. Wieght 8.4 kgm. Pancreatectomy 5–9–35. After recovery this animal was given the standard diet and from 5–12 to 6–16 received 100 to 600 cc. of pancreatic juice and 10 units of insulin daily. During this period the sugar excretion varied between 0 and 4.8 grams per day with an average of 1.2 grams. From 6–17 to 7–1 the juice was discontinued and during this period the sugar excretion varied between 0 and 9 grams with an average of 2.0 grams. From 7–1 to 7–9 400 cc. of juice was given and the sugar excretion averaged 2.8 grams on the standard diet and 10 units of insulin daily.

Dog 6. Male. Weight 15.1 kgm. Pancreatectomy 6–20–35. After recovery the animal received the standard diet and 20 units of insulin daily but from 6–20 until

7–8 was given no pancreatic juice. During this period the sugar excretion varied between 0 and 35 grams with an average of 11.0 grams per day. From 7–9 to 7–16 400 cc. of pancreatic juice were given daily in addition to the same dose of insulin and the same diet. During this period the sugar excretion varied between 1.0 and 40.0 grams with an average of 18.0 grams. From 7–17 to 7–31 no pancreatic juice was given and the sugar excretion varied between 0 and 7 grams with an average 1.0 grams per day.

Discussion. The results in both types of experiment were quite definite. In no instance did the continued total loss of pancreatic juice produce hyperglycemia or glycosuria in the dog. It is difficult to account for the reported findings of Boldyreff (1934) other than that this investigator must have confused the symptoms of dehydration and acidosis, resulting from the uncompensated loss of the alkaline pancreatic secretion, with diabetes. The data with respect to the feeding of pancreatic juice were equally clear cut. Depancreatized dogs 1, 2 and 3, receiving from 100 to 500 cc. of fresh pancreatic juice per day were in a somewhat better nutritional state than control animals, their stools were less bulky and more firm, but they remained diabetic and excreted large amounts of sugar on 15 to 30 units of insulin per day. The variation in the amount of sugar excreted by the depancreatized dog from day to day while on a constant diet and insulin intake was marked, in some cases amounting to as much as 45 grams. For this reason it was concluded that a comparison of the sugar excretion for short periods while pancreatic juice was administered would be misleading. In the protocols of dogs 4, 5 and 6, however, a comparison of periods of from 6 to 18 days was made, in each case a period of pancreatic juice feeding alternating with a control. The results were consistent throughout. In each case the administration of pancreatic juice not only did not decrease the sugar excretion but actually increased it. This effect would appear to be easily explained by the better digestion and absorption of glucose-forming substances in the intestine when pancreatic juice was given.

These observations are not out of harmony with the conclusions drawn by La Barre, Baxter, and Babkin from their experiments. Hyperglycemia may stimulate the secretion of pancreatic juice, but such increased secretion would not counteract the hyperglycemia if it were unaccompanied by an increased liberation of insulin into the blood stream.

SUMMARY

1. Total external pancreatic fistulae were prepared in six dogs. The complete withdrawal of pancreatic juice for 4 to 6 weeks did not cause hyperglycemia or glycosuria in these animals.

2. The oral administration of fresh dog pancreatic juice in amounts of from 300 to 1100 cc. per day to completely depancreatized dogs did not lessen the severity of the diabetes. On a standard diet and insulin intake,

the administration of pancreatic juice usually increased the glucose excretion.

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THE RELATION OF PANCREATIC JUICE TO THE FATTY INFILTRATION AND DEGENERATION OF THE LIVER IN THE DEPANCREATIZED DOG[1]

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Shortly after the discovery of insulin by Banting and Best (1922), it was noted by Fisher (1924) and by Allan, Bowie, Macleod and Robinson (1924) and others that completely depancreatized dogs adequately treated with insulin usually failed to survive more than two to three months. At death the most obvious change observed was an extensive fatty infiltration and degeneration in the liver. The addition of raw pancreas to the diet was found by the latter investigators to prevent the development of these liver changes and to permit survival for long periods of time. These findings have been abundantly confirmed. In 1930, Hershey, and in 1931, Hershey and Soskin, reported that the addition of 10 grams of lecithin daily to the diet of the depancreatized dog treated with insulin was also effective in preventing the liver damage and in permitting survival. The active constituent of lecithin in this effect was found by Best and Huntsman (1932) and Best, Ferguson and Hershey (1933) to be choline. Ralli, Flaun and Banta (1935) confirmed these observations with respect to lecithin, but concluded that it was not as effective as raw pancreas in preventing the deposition of liver fat. Berg and Zucker (1931) reported changes in the liver following pancreatic fistulae or ligation of the pancreatic ducts which they considered similar to those described by Fisher and by Allan, Bowie, Macleod and Robinson following pancreatectomy. They suggested that absence of the external pancreatic secretion from the intestine might be the common underlying factor in the three conditions.

The depancreatized animal clearly suffers from two known deficiencies, insulin and pancreatic juice. The fact that insulin together with an otherwise adequate diet does not suffice to permit the depancreatized animal to survive in good health, suggested that the pancreatic secretion might be of significance in this connection. The possibility that some of the pancreatic lipase might be absorbed into the blood and play a rôle in the migration of fat was considered by Allan, Bowie, Macleod, and Robinson, and led them

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to investigate the effect of feeding raw pancreas. The following experiments were done to determine if the beneficial effect of raw pancreas administration was due to its content of the enzymes or other substances present in pancreatic juice.

a. *The effect of the total loss of pancreatic juice on the liver.* If the deficiency in depancreatized dogs which leads to fatty changes in the liver and ultimately to death were the absence of one or more of the constituents of pancreatic juice, as for instance lipase, in the intestinal tract, then such changes might be expected to develop after total pancreatic fistula. This should be true unless it were assumed that the responsible substance was absorbed directly into the blood as well as secreted with the pancreatic juice. Observations were made on seven dogs in which total pancreatic fistulae were prepared as described in our previous paper (Harms, Prohaska and Dragstedt, 1936). They were given a standard diet of meat, bread, and whole milk, and dehydration and acidosis were controlled by the intravenous injection of Ringer's solution and the oral administration of alkalies. They secreted from 500 to 1,400 cc. of pancreatic juice per day with an average daily secretion of about 750 cc. Two of the animals developed extensive subcutaneous abscesses and were sacrificed after 30 and 36 days respectively. In both of these animals a moderate fatty infiltration of the liver was demonstrated in microscopic sections (fig. 1). One animal died from peritonitis following rupture of the duodeno-pancreatic pouch 23 days after operation. In this animal also a slight to moderate fatty infiltration of the liver was found. The remaining four animals were free from infection when sacrificed 26, 35, 39 and 43 days after operation. In each case the liver was found to be entirely normal, both on gross and microscopic examination.

b. *The effect of ligation of the pancreatic ducts on the liver.* Complete obstruction to the pancreatic ducts not only prevents the entrance of pancreatic juice into the duodenum, but has been demonstrated to cause an extensive degeneration of the acinar tissue of the pancreas. The islet tissue in many cases remains sufficiently functional, however, to prevent the appearance of diabetes. If the deficiency in question were due to the absence of some substance manufactured by the acinar tissue and absorbed directly into the blood, fatty changes might be expected to develop in the liver of such animals. In three adult dogs the pancreatic ducts were ligated and divided, the pancreas separated from the duodenum and the omentum interposed between to prevent regeneration or anastomosis of the duct system and the duodenum. These animals were given a generous diet of lean meat, whole milk and bread, but they rapidly lost weight and died in 44, 56 and 83 days respectively. At autopsy all of the animals were emaciated and chronic gastric ulcers were found in two. The pancreas in each case was extensively degenerated but some acinar tissue as well as the

islets were preserved. Sections of the liver showed slight fatty infiltration in two cases (fig. 2), but in the third the liver was entirely normal.

c. *Effect of the oral administration of pancreatic juice on the development of fatty degeneration and infiltration of the liver in depancreatized dogs.* Nine adult male dogs were completely depancreatized in a one-stage operation under ether anesthesia. Immediately after recovery they were placed on the standard diet consisting of 400 grams of meat, 100 grams of bread and

Fig. 1. Photomicrograph showing the slight degree of fatty infiltration in the liver of a dog who had a total pancreatic fistula for 36 days. Stain Sharlach R. Magnification ×235.

Fig. 2. Photomicrograph showing the slight degree of fatty infiltration in the liver of a dog whose pancreas had become extensively degenerated as a result of ligation of the pancreatic ducts. Stain Sharlach R. Magnification ×235.

Fig. 3. Photomicrograph showing the extreme degree of fatty infiltration and degeneration in the liver of a depancreatized dog treated with insulin and given 1000 cc. of pancreatic juice daily. Death occurred in 65 days. Specimen taken at autopsy. Stain Sharlach R. Magnification ×235.

Fig. 4. Photomicrograph showing the extreme degree of fatty infiltration and degeneration in the liver of a depancreatized dog treated with insulin and given 800 cc. of pancreatic juice daily. Death occurred 75 days after the pancreatectomy. Stain hematoxylin and eosin. Magnification ×235.

Fig. 5. Photomicrograph of a biopsy of the liver of a depancreatized dog taken 30 days after the pancreatectomy and showing moderate fatty infiltration. Stain Sharlach R. Magnification ×375.

Fig. 6. Photomicrograph of a biopsy of the liver of the same animal as used in figure 5 and showing the curative effect of feeding 25 grams of raw pancreas daily for one month. Stain Sharlach R. Magnification ×375.

Fig. 7. Photomicrograph of a biopsy of the liver of the same dog used in figure 6 and showing the return of fat in the liver after feeding only 10 grams of raw pancreas per day for one month. Stain Sharlach R. Magnification ×235.

Fig. 8. Photomicrograph of a biopsy of the liver of a depancreatized dog treated with insulin and showing definite fatty infiltration one month after the pancreatectomy. Stain hematoxylin and eosin. Magnification ×235.

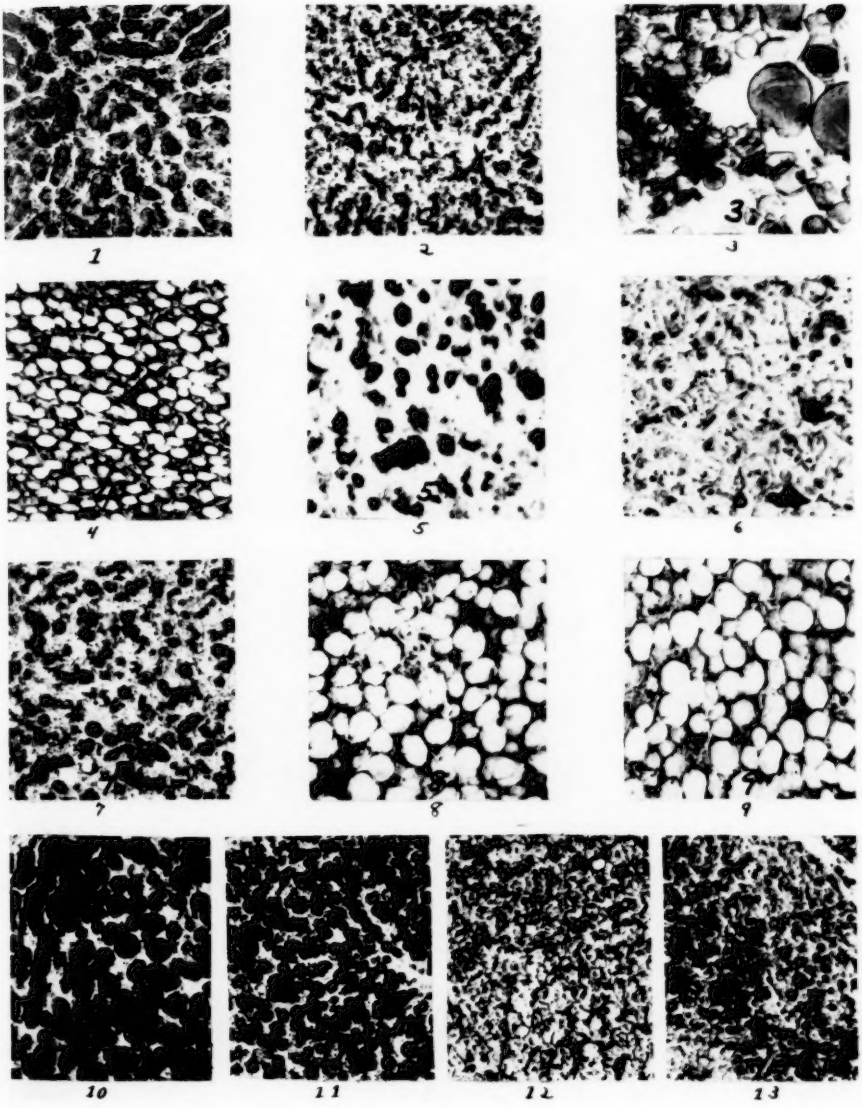
Fig. 9. Photomicrograph of a biopsy of the liver of the same dog used in figure 8 and showing an increase in the severity of the liver damage after the oral administration of 700 mgm. of choline chloride daily for 26 days. Stain hematoxylin and eosin. Magnification ×235.

Fig. 10. Photomicrograph of a biopsy of the liver of a depancreatized dog showing definite fatty infiltration 22 days after the pancreatectomy. Stain Sharlach R. Magnification ×180.

Fig. 11. Photomicrograph of a biopsy of the liver of the same dog used in figure 10 and showing an increase in the severity of the liver damage following the feeding of 100 grams of fresh beef brain daily for one month. Stain Sharlach R. Magnification ×180.

Fig. 12. Photomicrograph of a biopsy of the liver of a depancreatized dog showing moderate fatty infiltration. Stain Sharlach R. Magnification ×235.

Fig. 13. Photomicrograph of a biopsy of the liver of the same dog used in figure 12 and showing an increase in fatty infiltration after feeding 100 grams of raw beef brain daily for one month. Stain Sharlach R. Magnification ×235.



Figs. 1 to 13

400 cc. of whole milk. Insulin was administered twice daily and an attempt was made to adjust the dose so as to permit of only a moderate glycosuria. From 20 to 30 units daily were usually required. Fresh activated pancreatic juice usually obtained the same day from pancreatic fistula dogs was fed or given by stomach tube when not taken voluntarily. The amounts given varied between 100 and 1,000 cc. per day. Eight of the animals died during the course of the experiment, the length of survival after the pancreatectomy being 24, 41, 45, 56, 65, 68, 75 and 142 days respectively. In each case at autopsy an extreme degree of fatty degeneration and infiltration of the liver was found. The ninth animal also developed a fatty liver as revealed by biopsy after two months of pancreatic juice administration, and was then used for other studies. Only one of these animals displayed jaundice as a symptom of the liver damage. For the most part the symptoms in the order of their appearance were as follows: a decreased excretion of sugar in spite of constant food intake and insulin administration, increased sensitivity to insulin so that a dose previously well tolerated now caused convulsions, loss of appetite, loss of weight, apathy and muscular weakness. Microscopical sections showing the extensive liver changes in two representative animals appear in figures 3 and 4.

d. *The amount of fresh raw pancreas required to relieve and prevent fatty changes in the liver of the depancreatized dog.* Pancreas contains both lecithin and choline and a further study was now made to determine if these substances could account for the pancreas effect. As mentioned above it has been demonstrated by many workers that the addition of 100 grams of raw pancreas daily to the diet of the depancreatized dog of average size is sufficient when combined with insulin in adequate doses to permit survival in good condition for several years. It seemed important to determine if perhaps a smaller amount of fresh pancreas might suffice, and to secure such data the following experiment was done.

Dog 7. Male. Weight 7.6 kgm. Pancreatectomy 9–19–35. For the first month this animal received 400 grams of meat, 100 grams of bread, 400 cc. of whole milk, 400 cc. of pancreatic juice and 20 units of insulin daily. On 10–18–35 a biopsy of the liver was taken which showed a moderate degree of fatty infiltration (fig. 5). He was then given 25 grams of fresh raw beef pancreas daily in addition to the former diet except that the pancreatic juice was discontinued. A second biopsy of the liver taken on 11–22–35 showed marked improvement (fig. 6). On 12–3–35 the amount of raw pancreas fed was reduced to 10 grams per day, and a third biopsy taken 12–26–35 showed a return of the fatty change in the liver (fig. 7). It would thus appear that for a depancreatized dog weighing about 7 kgm. on a diet such as the standard one used in our experiments, 25 grams of fresh raw beef pancreas is enough to relieve the fatty infiltration of the liver but that 10 grams daily is too small a dose. The amount of choline in the rat pancreas was found by Fletcher, Best and Solandt (1935) to be in the neighborhood of 232 mgm. per 100 grams of pancreas. The amounts of choline, however, which MacLean and Best (1935) found to be effective in preventing or

relieving the fatty changes in the liver of the depancreatized dog varied between 1.5 and 2.25 grams per day. The effective dose of choline was found to be somewhat proportional to the amount of fat in the diet. It does not appear that Best and his associates have made a special effort to determine the minimum effective dose of choline for the depancreatized dog, and since this may vary depending on the diet it seemed desirable to us to determine the minimum dose required under the conditions of our experiments and to compare that with the amount that could possibly be present in an effective dose of raw pancreas.

e. *The amount of choline required to prevent or relieve fatty changes in the liver of the depancreatized dog.* Experiments were performed on four depancreatized dogs kept under standard conditions of diet and insulin administration and given respectively 200, 500, 700 and 1,000 mgm. of choline chloride by mouth per day. The data are summarized in the following protocols:

Dog 9. Male. Weight 12.6 kgm. Pancreatectomy 10–17–35. On 11–12–35 a biopsy of the liver was made and this showed a marked degree of fatty change. Two hundred milligrams of choline chloride were then given daily by mouth in addition to the standard diet. A second biopsy of the liver was made on 12–10–35, and this showed an even more extensive fatty infiltration than the first.

Dog 10. Male. Weight 14.5 kgm. Pancreatectomy 12–11–35. Biopsy on 12–30–35 showed a moderately fatty liver. Five hundred milligrams of choline chloride was then given for 9 days, but without clinical improvement. One gram of choline was given on the 10th day and 2.5 grams on the 11th day, but the animal became progressively worse and died on 1–12–36. Autopsy disclosed an extreme degree of fatty change in the liver.

Dog 11. Male. Weight 12.2 kgm. Pancreatectomy 1–17–36. Diet, 300 grams lean beef, 75 grams bread, 400 cc. of whole milk, and 20 units of insulin daily. Biopsy on 2–18–36 disclosed a moderate fatty infiltration of liver (fig. 8). Seven hundred milligrams of choline chloride was then given orally for 26 days. A second biopsy on 3–16–36 disclosed a more severe degree of fatty change in the liver (fig. 9).

Dog 12. Male. Weight 9.4 kgm. Pancreatectomy 12–12–35. Standard diet plus 10 units of insulin daily. Biopsy on 12–30–35 disclosed a slight fatty infiltration of the liver. One gram of choline chloride was then given orally for 14 days at which time the animal developed pneumonia and died. At post-mortem examination microscopic sections showed definite regeneration of the liver.

The results of this experiment indicate that whereas 1.0 gram of choline chloride daily was sufficient to improve the condition of the liver of the depancreatized dog, smaller doses such as 200, 500 or even 700 mgm. per day were entirely ineffective. Thus while we were able to secure a definite effect with smaller doses of choline than hitherto reported, the minimum amount required was still approximately 15 times as much as is calculated to be present in 25 grams of fresh pancreas, an effective dose of this substance.

f. *The effect of the oral administration of fresh beef brain on the fatty changes in the livers of depancreatized dogs.* Lecithin and choline are present not only in pancreas but in even greater amounts in certain other tissues.

Thus Fletcher, Best, and Solandt (1935) found the brain of the rat to contain approximately 325 mgm. of choline per 100 grams of tissue. Since it was clear that the pancreatic enzymes played no rôle in the beneficial effect of pancreas feeding, experiments were done to determine the effect of the oral administration of comparable amounts of fresh beef brain.

Dog 8, a male, weighing 12 kgm. was depancreatized 12–4–35 and placed on the standard diet and 15 units of insulin daily. A biopsy taken 22 days later on 12–26–35 disclosed a definite fatty infiltration of the liver (fig. 10). One hundred grams of fresh raw beef brain were added to the diet from 12–31–35 to 1–30–36 when a second biopsy was taken. This showed a much more marked fatty degeneration and infiltration of the liver (fig. 11). A similar result was obtained with dog 9 that had been used before in an experiment with choline and with pancreas. This animal, a male weighing 12.6 kgm., was depancreatized 10–17–35, but as a result of treatment was in good condition on 2–3–36 when a biopsy of the liver disclosed only moderate fatty infiltration (fig. 12). One hundred grams of fresh raw beef brain were added to the diet from 2–7–36 to 3–3–36 when another biopsy of the liver were taken. This showed a marked increase in the extent of the liver damage (fig. 13).

g. *The effect of the oral administration of fresh beef liver on the fatty changes in the liver of the depancreatized dog.* The negative results obtained by the feeding of fresh brain tissue suggested that the effect obtained with fresh pancreas was specific. To check this impression further, a trial was now made with fresh beef liver. A depancreatized dog that had developed a fatty liver 27 days after pancreatectomy and had been relieved by pancreas administration was selected for the experiment. A preliminary biopsy of the liver was taken and this revealed definite fatty changes. The animal weighed 11.0 kgm. and on the standard diet and 20 units of insulin daily excreted an average of about 18 grams of sugar in 24 hours. One hundred grams of fresh raw beef liver were then administered daily in addition to the usual diet and insulin. After 12 days of liver feeding the sugar excretion decreased and the insulin dosage was reduced to 15 units. Twenty-four days after the beginning of liver feeding symptoms of liver damage became pronounced and a biopsy revealed a marked increase in the extent of fatty infiltration and degeneration in the liver. During the last 12 days of the experiment the average daily sugar excretion was less than 1 gram although the animal consumed all of his food and received only 15 units of insulin per day.

Discussion. The results obtained in the experiments described above indicate very definitely that the absence of pancreatic juice from the intestine is not the cause of the fatty changes in the liver of the depancreatized dog. The slight degree of fatty infiltration of the liver observed in three of the pancreatic fistula dogs was by no means comparable to the extensive changes seen after pancreatectomy. Furthermore, the fact that this slight fatty infiltration occurred only in the three fistula animals which developed severe infections, whereas the other four had normal livers

suggests that the infection rather than the loss of the pancreatic secretion is the responsible factor. It is probable that a similar explanation may account for the liver changes observed by Berg and Zucker in the pancreatic fistula dogs prepared by cannulating the ducts.

The changes in the livers of two of the animals in which the pancreatic ducts had been ligated were so slight as compared with those after pancreatectomy that we consider the effect negative. The liver of the third animal was normal. These observations suggest that it may not be the loss of acinar tissue which causes the fatty changes in the liver after pancreatectomy, but that this is due to a deficiency in islet function. A definite statement cannot be made since although the great bulk of the acinar tissue of the pancreas in these cases was degenerated, small remnants of apparently normal cells were still present in the periphery of many acini.

The depancreatized dogs given pancreatic juice as a supplement to insulin treatment lived no longer than control animals on insulin treatment alone. Furthermore, the degree of fatty degeneration and infiltration of the liver and the rapidity of its onset after pancreatectomy were either not affected by the administration of the juice or the condition was aggravated. The secretion was given daily in amounts equal to those normally secreted and with only short intermissions for the entire post-operative period. The digestion and absorption of food in the intestines was markedly improved, as evidenced by the increased sugar excretion and the decrease in the bulk of the feces, but if anything, the accumulation of fat in the liver was hastened rather than retarded. It is thus very evident that the beneficial effect of raw pancreas is not due to the pancreatic enzymes it contains or to an improvement in the digestion and absorption of fats occasioned by the presence of those enzymes.

While the observations of Best and his associates that choline will relieve the fatty degeneration of the liver of the depancreatized dog have been confirmed, the amount required was so great that it does not seem possible to account for the pancreas effect on this basis. We were unable to secure a favorable response with less than 1.0 gram of choline chloride daily, whereas the amount of choline in a minimum adequate dose of fresh pancreas (25 grams) we have estimated to be about 60 mgm. The fact that the administration of raw brain or liver (100 grams daily) was unable to prevent or relieve the characteristic fatty changes in the liver is significant since brain contains somewhat more lecithin and choline than is found in pancreas. While it is altogether probable that these substances play an exceedingly important rôle in the transport and metabolism of fat, we believe that the evidence presented is more in harmony with the view that there is some other specific substance in pancreas, whose absence leads to the liver changes described, and whose presence in the fresh pancreas is chiefly responsible for its beneficial effect when fed to depancreatized animals.

CONCLUSIONS

1. The fatty degeneration and infiltration of the liver, which occurs in depancreatized dogs treated with insulin, is not due to the absence of pancreatic juice from the intestines since:

a. It does not occur in dogs provided with total pancreatic fistulae.

b. It does not develop in dogs following ligation of all pancreatic ducts and degeneration of pancreatic parenchyma.

2. The beneficial effect of raw pancreas feeding after pancreatectomy is not due to the pancreatic enzymes since the administration of fresh pancreatic juice has no such beneficial effect.

3. Choline and lecithin are probably not the substances in pancreas which are responsible for its effect in prolonging the survival of depancreatized dogs treated with insulin and in preventing the fatty changes in the liver, since:

a. The minimum effective dose of choline in relieving the fatty changes in the liver of the depancreatized dog is many times greater than the amount present in an effective dose of pancreas.

b. Fresh raw brain has no such beneficial effect, although it contains more lecithin and choline than pancreas.

4. The beneficial effect of raw pancreas feeding after pancreatectomy is probably due to some specific substance in pancreas since equivalent amounts of liver or brain were ineffective in this respect.

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OBSERVATIONS ON A SUBSTANCE IN PANCREAS (A FAT METABOLIZING HORMONE) WHICH PERMITS SURVIVAL AND PREVENTS LIVER CHANGES IN DEPANCREATIZED DOGS[1]

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The early report of Fisher (1924), Allan, Bowie, Macleod, and Robinson (1924), and the subsequent extensive studies of Best and his associates (1932, 1933, 1934, 1935) demonstrated quite conclusively that the completely depancreatized dog would not survive more than a few months even though adequately treated with insulin. The most prominent abnormality seen at autopsy was an extreme degree of fatty degeneration and infiltration in the liver. These changes in the liver could be prevented and life indefinitely prolonged by the addition of adequate amounts of raw pancreas, lecithin, or choline to the diet. An exception to these statements is found in the report of Chaikoff (1935) that he has maintained two depancreatized dogs alive and in good condition for over four years by means of insulin but without the administration of pancreas, lecithin, or choline. These probably represent exceptional instances. Furthermore, both animals were still living at the time of the report so that verification of the completeness of the pancreatectomy is not yet available. In the present studies we have depancreatized 45 dogs and all save one developed the characteristic changes in the liver as verified by biopsy or post-mortem examination. Eight of these animals received no supplement to the diet, which was effective in relieving the fatty changes in the liver, and all died very promptly. In the great majority of cases the animals remained in good condition for three to four weeks after the pancreatectomy, excreting considerable amounts of sugar on a diet of 400 grams of meat, 400 cc. of whole milk, and 100 grams of bread with 20 to 30 units of insulin daily. Then the sugar excretion began to diminish, the insulin requirement to decrease, and the animals became apathetic and more and more indifferent to food. Jaundice was rarely observed. Biopsy of the liver taken during the presence of these symptoms invariably revealed an extensive degree of fatty change. We were soon convinced that animals manifesting these symptoms would shortly

[1] This work has been aided by a grant from the Douglas Smith Foundation for Medical Research of The University of Chicago.

die unless some effective supplement was added to the diet. Only one of the 45 animals failed to develop a fatty liver, but this one died 72 days after the pancreatectomy and no significant changes other than the pancreatectomy were found at post-mortem examination.

The findings described in the preceding paper, indicating that the beneficial effect of raw pancreas in preventing and relieving the fatty changes in the liver of the depancreatized dog could not be accounted for on the basis of its lecithin or choline content or by the presence of the pancreatic enzymes, suggested that the effect must be due to some other substance. An attempt was made to secure such a substance in various types of pancreatic extracts. Fractionation of the pancreas was difficult and time consuming because of the nature of the criteria involved. In every instance the activity of a fraction was tested by its ability to relieve already established fatty degeneration and infiltration in the liver of the depancreatized dog. To accomplish this a number of dogs were carefully depancreatized, placed on our standard diet of 400 grams of meat, 400 cc. of whole milk, and 100 grams of bread, and given sufficient insulin daily to permit of only a moderate glycosuria. After a period of four weeks symptoms suggestive of fatty liver usually appeared and a control biopsy of the liver was taken. If this showed definite fatty infiltration the animal was given one of the pancreas extracts mixed with the food. Usually an amount of extract obtained from 100 grams of fresh pancreas was employed and this dose was given daily for a period of 3 to 6 weeks and sometimes longer. A beneficial effect was occasionally manifested in a few days where the fatty infiltration was very marked and the condition of the animal poor; this might be delayed for a week or longer. The symptoms of such improvement were usually increase in appetite, increase in sugar excretion, and renewed activity. In every instance a second biopsy was taken after 3 to 4 weeks and the conclusion as to the activity of the extract tested was based largely on a comparison of the microscopic appearance of the two biopsy specimens. This method proved very reliable since the fatty changes in the liver were found to be practically uniform throughout and improvement following the administration of active preparations equally diffuse and widespread.

Experimental procedure. Fresh beef or calf pancreas, received usually in a partially frozen state, was stripped of adherent fat, finely minced, and mixed with about two volumes of 95 per cent ethyl alcohol. The mixture was stirred frequently and allowed to extract for five to six hours. The alcohol was then filtered off and the residue treated three more times in similar fashion with 95 per cent alcohol. The alcoholic filtrates were then combined and evaporated in shallow pans at room temperature to a thick brown paste. This was then extracted from two to five times with several volumes of sulphuric ether. The residue was

found to be soluble in 5 per cent NaCl solution and in water. This portion was called the "fat-free alcohol extract" and from 1.8 to 2.5 grams of the dried product was obtained from 100 grams of fresh pancreas. The residue from the original alcohol extractions was then dried and extracted several times (2 to 5) with ether. The various ether filtrates from this extraction and from the extraction of the alcohol extract were combined and evaporated. This residue, which contained practically all of the lipids of the original pancreas, was called the "ether extract." From 10 to 14 grams of this dried ether extract were obtained from 100 grams of fresh pancreas. The residue of pancreas remaining after both alcohol and ether extraction was called the "pancreatic residue." This residue was extracted with 5 per cent NaCl solution, the extract filtered off and labeled "salt solution extract of pancreas residue." In many instances the effect of various extracts was tested alternately on the same animal so as to secure the advantage of comparison and for purposes of control. The results are indicated in the following representative experiments.

Dog 7. Male. Weight 7.6 kgm. Pancreatectomy 9–19–35. Biopsy 10–18–35 showed a fatty liver; 25 grams of raw pancreas were then added to the diet and a second biopsy 11–22–35 disclosed an almost normal liver. The dose of pancreas was then reduced to 10 grams and a third biopsy 12–26–35 indicated a return of fat in the liver (fig. 1). Fourteen grams of "ether extract" of pancreas (the amount obtained from 100 grams of fresh gland) were then fed instead of the pancreas for a period of 19 days and a fourth biopsy 1–14–36 showed a marked increase in the degree of fatty change in the liver (fig. 2). The animal was then given 1.5 grams of the "fat-free alcohol extract" (the amount obtained from 100 grams of fresh gland) and a fifth biopsy 2–18–36 after 34 days administration showed a definite improvement in the appearance of the liver (fig. 3).

Dog. 9. Male. Weight 12.6 kgm. Pancreatectomy 10–17–35. This animal developed a fatty liver within one month and this was not relieved but became more marked during the second month while it was receiving 200 mgm. of choline daily as supplement. Biopsy taken on 12–10–35 revealed a very fatty liver (fig. 4). The "fat-free alcohol extract" was then given instead of the choline and a biopsy taken 20 days later 12–30–35 showed some improvement in the liver. The administration of the extract was continued and the condition of the animal improved markedly. Another biopsy taken 2–3–36 showed almost complete recovery (fig. 5). This animal later developed a fatty liver again while given raw brain as a supplement.

Dog 10. Weight 8.6 kgm. Pancreatectomy 11–11–35. A biopsy taken 12–6–35 showed fatty infiltration in the liver (fig. 6). The animal was then fed "pancreatic residue" (amount corresponding to 100 grams of original pancreas) as a supplement and a second biopsy taken 1–7–36 showed definite improvement (fig. 7). The "fat-free alcohol" extract was then given for 27 days and a third biopsy 2–3–36 showed still more recovery. Both the "fat-free alcohol" extract and "salt solution extract of pancreas residue" were then given for 30 days and a fourth biopsy taken 3–4–36 disclosed further improvement (fig. 8).

Dog 12. Male. Weight 8.8 kgm. Pancreatectomy 12–31–35. A biopsy of the liver taken 1–17–36 showed definite fatty infiltration of the liver (fig. 9). "Salt solution extract of pancreas residue" in an amount corresponding to 125 grams of fresh pancreas was then fed for one month and a second biopsy taken 2–18–36 disclosed almost complete regeneration of the liver (fig. 10).

Dog 13. Weight 916 kgm. Hypophysectomy 11–19–35. On 12–3–35 the general condition was good and the blood sugar was found to be 67 mgm. per 100 cc. Pancreatectomy was done on 12–3–35. The animal was then placed on the standard diet but given no insulin. The sugar excretion at first ranged between 5 and 12 grams per day but gradually decreased and after 3 weeks the urine became practically sugar free. A biopsy of the liver taken 1–7–35, 5 weeks after the pancreatectomy, revealed marked fatty changes (fig. 11). One and five-tenths gram of the "fat-free alcohol extract" was then given daily for about 6 weeks and a second biopsy on 2–19–36 showed definite improvement in the liver (fig. 12). During the last 3 weeks of this period the sugar excretion increased to an average of 28 grams per day. No insulin was administered at any time.

Observations were made on nine additional dogs besides those described in the protocols. The results were strikingly uniform throughout. The "ether extract" of pancreas containing the lipid fraction was in no case found to be active but rather seemed to increase the rate of fat deposition

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Fig. 1. Photomicrograph showing a moderate degree of fatty infiltration in the liver of a depancreatized dog. Stain Sharlach R.

Fig. 2. Photomicrograph of a biopsy of the liver of the same animal as in figure 1 and showing a definite increase in the degree of fatty change after the administration of the "ether extract" of pancreas for 19 days. Stain hematoxylin and eosin.

Fig. 3. Photomicrograph of a biopsy of the liver of the same animal as in figures 1 and 2 and showing a marked improvement in the liver after the administration of the "fat-free alcohol extract" of pancreas for 34 days. Stain hematoxylin and eosin.

Fig. 4. Photomicrograph of a biopsy of the liver of a depancreatized dog showing a marked degree of fatty infiltration. Stain Sharlach R.

Fig. 5. Photomicrograph of a biopsy of the liver of the same animal as in figure 4 and showing the disappearance of the fat after the administration of the "fat-free alcohol extract" for 53 days. Stain Sharlach R.

Fig. 6. Photomicrograph of a biopsy of the liver of a depancreatized dog showing a moderate degree of fatty change. Stain Sharlach R.

Fig. 7. Photomicrograph of a biopsy of the liver of the same animal as in figure 6 and showing some improvement produced by the administration of "pancreatic residue" for 30 days. Stain Sharlach R.

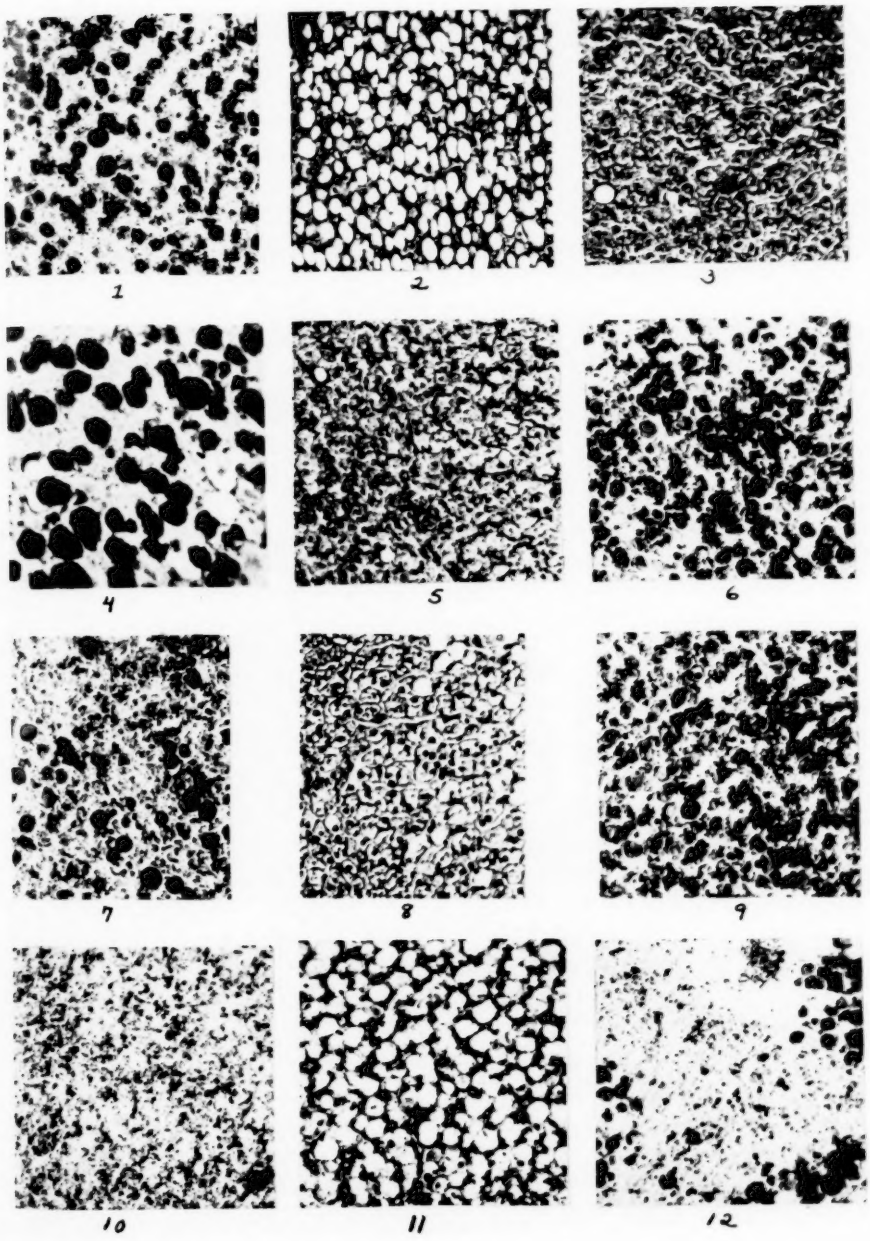
Fig. 8. Photomicrograph of a biopsy of the liver of the same animal as in figures 6 and 7 and showing complete recovery after the administration of "fat-free alcohol extract" and "salt solution extract of pancreas residue" for 57 days. Stain hematoxylin and eosin.

Fig. 9. Photomicrograph of a biopsy of the liver of a depancreatized dog showing marked fatty infiltration. Stain Sharlach R.

Fig. 10. Photomicrograph of a biopsy of the liver of the same animal as in figure 9 and showing the disappearance of fat after the administration of the "salt solution extract of pancreas residue" for one month. Stain Sharlach R.

Fig. 11. Photomicrograph of a biopsy of the liver of a hypophysectomized-depancreatized dog showing extreme fatty changes. Stain Sharlach R. The fat droplets have not stained deeply.

Fig. 12. Photomicrograph of a biopsy of the liver of the same animal as in figure 11 and showing the improvement produced by the administration of the "fat-free alcohol extract" of pancreas for six weeks. Stain Sharlach R.



Figs. 1 to 12

in the liver. The "fat-free alcohol extract" of pancreas was in each case found to contain the active principle and to be effective in relieving already established fatty liver in depancreatized dogs when given in a daily dose of from 1.0 to 1.5 gram of the dried substance. This was somewhat less than the amount derived from 100 grams of fresh gland. The "pancreatic residue" and the "salt solution extract of the pancreatic residue" were each found to contain the active principle when the original alcohol extractions were carried out with 95 per cent alcohol but in greatly reduced amount when 60 per cent alcohol was used. Apparently the lower concentration of alcohol was a better solvent and removed most of the substance from the pancreas.

Observations were made on one additional hypophysectomized-depancreatized dog besides the one mentioned in the protocols. Neither of these animals received insulin at any time. Both developed the characteristic changes in the liver just as rapidly as the depancreatized dogs in our series and they responded quite as well when given the active extracts.

The facts that have appeared as a result of this study would seem to warrant the conclusion that there is present in fresh pancreas a specific substance, aside from lecithin or choline, which is effective on oral administration in preventing or relieving the fatty degeneration and infiltration of the liver in depancreatized dogs. This substance is insoluble in ether, but soluble in alcohol, 5 per cent NaCl solution, and in water. The fact that it is not present in pancreatic juice makes it seem probable that it is a hormone which under normal conditions plays a rôle in the transport or further utilization of fat. It is possible that choline may play some important intermediary rôle in this function. Hypophysectomy, which lessens the necessity for insulin in conserving the life of the depancreatized dog, does not compensate for the absence of this fat metabolizing hormone.

After consultation with Carl D. Buck, Professor of Comparative Philology of The University of Chicago, we have chosen the name "lipocaic" for this substance. It is derived from the Greek words "λιπος," "fat," and "καια," "I burn." A more general term suggesting that the hormone plays a rôle in the utilization of fat was sought but without success.

CONCLUSIONS

A specific substance has been obtained in alcoholic extracts of beef pancreas, that on oral administration to depancreatized dogs treated with insulin, permits survival and prevents and relieves the fatty degeneration and infiltration of the livers of these animals. This substance, for which the name "lipocaic" is suggested, is believed to be a new hormone that is concerned in some way with the normal transport and utilization of fat.

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HISTONE COMBINATIONS OF THE PROTEIN HORMONES

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In searching for protein precipitants which might be used clinically to delay resorption of parenterally administered protein hormones, the histones, being well known as protein precipitants, presented themselves as more or less obvious possibilities. Trial in experimental animals of the insoluble thymus histone combination of insulin and of the pituitary gonadotropic preparation produced results entirely unexpected, differing from those previously obtained in this laboratory with the zinc, iron, and tannic acid combinations of these hormones (1) (2) (3) (4).

Preparation of histone. The thymus histone prepared according to the method of Kossel and Kutscher (5) did not in our hands yield a product completely acid soluble, and it contained a high ash content. The following modifications were introduced. The first ammonia precipitate, after washing with ammonia water, was taken up in 0.9 per cent HCl. Insoluble material was removed by filtration, after which the filtrate was neutralized with ammonia to a pH faintly pink to phenolphthalein. The slight mostly crystalline precipitate which formed was removed by centrifugation. (It analyzed 46 per cent ash.) The filtrate was then made strongly alkaline with ammonia. From this point the procedure of Kossel and Kutscher was again followed. The resulting product was acid soluble, contained 4 per cent ash[1] and 17.0 per cent nitrogen, gave positive biuret and Millon's tests, was precipitated by trichloracetic but not by nitric acid. A stock solution containing 10 mgm. per cubic centimeter was prepared by solution in dilute HCl. This was sterilized by heating to 70°C. on three successive days.

Insulin combination. From the isolectric point of insulin to a pH more alkaline than 8.5 the histone precipitates insulin,[2] and the resulting sus-

[1] Presumably magnesium ammonium phosphate. Negative H_2S group. Not ammonia soluble. Zinc is ruled out. The absence of zinc is of great importance to the interpretation of the results, since small amounts of zinc, peptized by insulin in mildly alkaline solution, precipitate the insulin at neutrality. The negative results with the pituitary gonadotropic preparation, which is very sensitive to the presence of zinc, corroborate the absence of this metal.

[2] Fortunately these studies were begun before the appearance of the recent statement of Hagedorn, Jensen and Krarup (J.A.M.A. **106**: 177, 1936) that insulin does not form an insoluble combination with histone; we doubtless would have taken the Danish workers at their word. These authors, using serum as a solute for solubility

pension, if standardized by intramuscular injection, shows a marked (at least 75 per cent) decrease in activity (table 1). If the suspension is given intravenously the activity approaches very nearly that of the original insulin. By giving massive doses of the histone precipitate intramuscularly it was found possible to produce prolonged hypoglycemia without producing insulin shock (table 2).

The procedures in testing the insulin were the same as those described in earlier publications (2) (4). In the experiments recorded 1.5 mgm.

TABLE 1

Comparison of intramuscular and intravenous injections of insulin-histone in rabbits

INSULIN PREPARATION	DOSE PER KILO	NUMBER OF RABBITS	ROUTE OF INJECTION	BLOOD SUGARS IN MILLIGRAMS PER 100 CC.		
				1 hour	3 hours	5 hours
Insulin	1 u	10	Intramuscular	46 ±3	51 ±5*	101
Histone-insulin	1 u	Same	Intramuscular	77 ±3	99 ±4	108
Insulin	1 u	9	Intravenous	50 ±4	59 ±6	89
Histone-insulin	1 u	Same	Intravenous	50 ±4	67 ±6	98

* Three rabbits in the controls convulsed. The blood sugar data for these rabbits are not incorporated. The effect is therefore even more striking than the data would indicate.

TABLE 2

Influence of massive intramuscular dosage of insulin-histone upon the blood sugar response in rabbits

(One-fourth the dose given as the histone in these experiments, produced insulin convulsions when given as ordinary insulin.)

RABBIT	BLOOD SUGAR IN MILLIGRAMS PER 100 CC.								
	0 hour	1 hour	3 hours	5 hours	8 hours	10 hours	12 hours	16 hours	24 hours
C	125		72		72	80		116	130
K	110	54	40	62	48		62	80	122
N	140	58	48	58	70		76	90	150
B	120	62	38	52	78	83			114
F	104	50	40	66	74	96			106

histone preparation was used for every 10 units of commercial insulin. This was more than double the amount required to completely precipitate the insulin. Experiments using 0.4 and 0.8 mgm. histone per 10 units insulin produced the same results as those recorded.

Prolan experiments. The prolan was prepared from urine of pregnancy by the conventional fractional alcohol precipitation procedure. The powder was completely water and 50 per cent alcohol soluble. The stand-

tests, failed to consider the remarkable peptizing powers of this medium (an error similar to that made some years back by the Harvard group studying lead salt solubility).

ardization was performed upon 22–23 day old rats of both sexes at five dosage levels using 24 rats at each dosage level, and ascertaining ovarian, seminal vesicle, and prostate weights and presence of corpora lutea. The preparation assayed 25 units per mgm. (Unpublished data of M. L. Long.)

Thymus histone-prolan combination. The addition of a solution of thymus histone to the prolan solution at pH 6.0 produced a precipitate, there being, however, no change in the activity of the prolan as measured by the effect upon ovarian, seminal vesicle, or prostate weight. See table 3. The following experiment indicated the potency was in the filtrate. Solutions of 100 mgm. prolan and 105 mgm. thymus histone were allowed to react at pH 6.0. The resulting precipitate was removed by centrifugation and washed twice with water. The combined filtrates were taken to pH 8, and alcohol added to 47 per cent concentration. The resulting precipitate was removed by centrifugation and washed twice with 47 per

TABLE 3

Effect of thymus histone upon prolan

PREPARATION ADMINISTERED	RECOVERY AS MEASURED BY:			Number of rats
	Ovarian weight	Seminal vesicle weight	Prostate weight	
	per cent	*per cent*	*per cent*	
Prolan + histone (1:5), pH 6.0	100	100	100	8
Prolan + histone (1:1):				
pH 6.0 precipitate	5	5	5	5
pH 8.2 precipitate	10	10	10	5
Filtrate, alcohol precipitate	50	75	50	5

cent alcohol. The combined filtrates were taken to pH 6.0 and alcohol added to 83 per cent concentration. The precipitate was removed by centrifugation. All precipitates were finally dissolved at pH 5.0 for assay. It will be noted (table 3) that only slight activity was manifested by the histone precipitates. Over half the active material was recovered from the filtrate (usual for an alcohol precipitation). The pH 6.0 precipitate was highly colored. The procedure could doubtless be used in the purification of prolan.

Pituitary gonadotropic preparation. The preparation studied was the same described in an earlier publication (6). The aqueous solution formed a precipitate with thymus histone (pH 6.0–8.0). The resulting product showed a slight decrease in activity as measured by ovarian weight (table 4). No activity was found in the histone precipitate. The fraction of activity remaining after histone treatment was in the filtrate. The data given in the table were for the ratio 1:2::histone:pituitary powder. Increase of the amount of thymus histone used to a ratio of 2:1 did not

produce complete inactivation, there being corpora lutea in the ovaries of the dosed animals.

In order to increase the sensitivity of the assay the experiment was repeated, adding Zn before dosage. The results indicated there was some activity in the histone precipitate (between 12 and 25 per cent) (table 4).

DISCUSSION. The results clearly show that the histone does not combine to form an insoluble compound with prolan and in no way influences the physiologic effect of prolan. In the case of the pituitary gonadotropic preparation most of the active material is not in the histone precipitate, but in the filtrate. On the basis of these findings an augmentation in activity would not be expected. A significant decrease in activity was observed when the assay was performed in the ordinary way while complete recovery of the activity was indicated when the preparations were assayed

TABLE 4

Influence of histone upon the pituitary gonadotropic preparation

PREPARATION ADMINISTERED (TOTAL DOSE PER RAT)	OVARIAN WEIGHT	NUMBER OF RATS
8 mgm. pituitary powder	29 ±2.4	10
8 mgm. pituitary powder + 4 mgm. histone	18 ±1.6	7
Precipitate of above histone combination	13 ±0.9	6
Filtrate of above histone combination	21 ±2.2	6
1 mgm. pituitary powder + 0.6 mgm. Zn	34	5
2 mgm. pituitary powder + 1.0 mgm. Zn	83	4
4 mgm. pituitary powder + 2.6 mgm. Zn	96	5
8 mgm. pituitary powder + 3.2 mgm. Zn	126	4
Precipitate + 2.6 mgm. Zn of 8:8 mgm. histone combination	67	6
Filtrate + 2.6 mgm. Zn of 8:8 mgm. histone combination	157	5

after the addition of Zn ion (1). The histone apparently combines with naturally occurring protein impurities which, in the conventional assay, serve to decrease the liberation of the active material from the tissue depots by themselves adsorbing the active material. The histone would therefore fill the requirements of those who allude to pituitary inhibitors, only in this case the source would be extrapituitary. On the basis of our findings it is unnecessary to require pituitary inhibitors to explain the physiological findings. It is now well established that the physiological activity of the pituitary gonadotropic extract is largely dependent on the rate of liberation from the tissues. So-called pituitary inhibitors are probably substances like histone, which accelerate liberation of the hormone. Their effect is the opposite of that of zinc or tannic acid which delay the liberation.

In the case of insulin, an insoluble combination with the histone exists

over the pH range found in the body tissues. Since the intravenous administration of this combination produces a hypoglycemic curve in no way differing from that of the original insulin, it may be definitely concluded that insulin is not inactivated by combination with the histone. The marked decrease in activity observed when the histone combination is given intramuscularly and assayed in the conventional manner is markedly in contrast to the results obtained with other insoluble insulin combinations such as the tannate, iron, and zinc combinations. The two former produce an augmentation in effect, the latter a prolongation of effect with a more shallow hypoglycemia curve. It is probable that the histone combination is broken up much more slowly than the other combinations mentioned, and the liberation of most of the insulin spread over such a long period that its hypoglycemic effect (giving 1 u per K) is not detectable in the normal animal. This is borne out by the prolonged hypoglycemia without insulin shock produced by increased dosage of the histone combination. These questions can best be answered by administration of the histone-insulin combination to the diabetic animal. Such studies, clinical in nature, will be presented elsewhere.

A brief summary of the clinical experience of Dr. P. A. Gray with our histone preparation is included.

Histone-insulin on clinical trial. To-date insulin-histone has been used in the treatment of human diabetes in 11 cases. In 3 patients, who have a mild form of diabetes, the defect in metabolism has been satisfactorily controlled by one hypodermic injection of insulin-histone per day. In the other cases the new insulin has been used on conjunction with regular insulin. In all instances to-date the new insulin has been found to be an adequate substitute for old or regular insulin. Its action is noticeable 24 hours after a single injection, but not after 48 hours. Clinical manifestations of hypoglycemic shock follow appropriate dosage. No local or systemic reactions have been encountered.

Case H-6 received one injection of insulin-histone (24 units) before breakfast for each of 4 consecutive days. The fasting blood sugar 24 hours later varied from 114 to 142 mg. per cent. A cross-section of one day follows:

TIME	INSULIN—H UNITS	BLOOD SUGAR	REMARKS	DIET
5:30 a.m.		106		C-343
5:40 a.m.	30			P-91
11:00 a.m.		90	Hypoglycemia	F-86
5:00 p.m.		112		
11:00 p.m.		98		
5:00 a.m.		114		

Insulin-histone is active upon hypodermic injection in human subjects as shown by (1) its ability to control human diabetes mellitus either when used alone or in conjunction with regular insulin, and (2) the production of hypoglycemic shock after appropriate dosage. When it is injected daily a "pooling effect" is noticeable after 4 to 5 days, necessitating reduction in the total daily unitage. The clinical effect of insulin-histone upon a case showing insulin resistance has been striking and will be reported elsewhere.

We are indebted to Miss M. Louisa Long and Mr. Russel Spicer for assistance in the biologic standardization and to Dr. P. A. Gray for his summary of his clinical findings.

SUMMARY

1. The thymus histone precipitates insulin on the alkaline side of the insulin isolectric point. Given intravenously this combination produces a blood sugar response approximating that of the original insulin. Given intramuscularly a marked decreased in activity is indicated when the assay is made in the conventional manner. Prolonged hypoglycemia without shock is produced by larger doses.

2. Material of low activity is separated from an active filtrate, when thymus histone is added to either prolan or pituitary gonadotropic preparations in the pH range 6.0–8.0. Histone by combining with the naturally contaminating proteins, which adsorb the pituitary gonadotropic principle, produces a decrease in physiological activity when the assay is made in the conventional manner. Complete recovery is indicated when zinc salts are added. The mechanism of the so-called pituitary inhibitors is thus explained.

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